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Assessment of the efficacy of *Piper betle* methanolic extract on water quality and immunity level in Tiger shrimp (*Peneaus monodon*)

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Abstract

The tiger shrimp farming industry is currently dealing with problems associated with infections and disease outbreaks, which cause huge losses in many countries. The current practice of disease prevention often utilizes antimicrobial agents and drugs that eventually pose drawbacks and raise some concerns for animal, human, and environmental health. Therefore, the enhancement of the non-specific immunity of tiger shrimp through the application of immunostimulants derived from medicinal plants has been found to be effective. *Piper betles* contain numerous bioactive constituents that contribute to a variety of biological actions, including antimicrobial, antioxidant, anti-inflammatory, and immunomodulatory actions. The present study aimed at investigating the effect of a methanolic extract of *Piper betle* on water quality and immunity levels in tiger shrimp through a bath immersion approach. Tiger shrimp (*Penaeus monodon*) were maintained in the artificial seawater at an optimum condition for shrimp breeding. The bath immersion experiment was conducted by maintaining four groups of tiger shrimp in water containing different concentrations of *Piper betle* extract (0.00 mg/L, 0.05 mg/L, 0.10 mg/L, and 0.15 mg/L). Water quality for each treatment was monitored on a daily basis based on parameters including pH, salinity, and content of ammonia, nitrate, and nitrite. The immunostimulatory effect of *Piper betle* in tiger shrimp was determined based on growth performance, analysis of haemoglobin levels, and total bacterial load. Analysis of water quality demonstrated significant improvements in pH, salinity, and content of ammonia, nitrate, and nitrite in the treated water incorporated with 0.15 mg/L of *Piper betle* extract. Growth performance analyses demonstrated enhancements in weight and length gained from the shrimp treated with 0.15 mg/L concentrations of *Piper betle* extract. The tiger shrimp maintained in water containing 0.15 mg/L concentration of *Piper betle* extract demonstrated the highest haemocyte count and the lowest bacterial load when compared to other treatment groups. This study demonstrated that the addition of 0.15 mg/L of *Piper betle* in artificial seawater is an optimum concentration that improves water quality and enhances growth performance and immunity levels in tiger shrimp.

Keyword: Piper betle; tiger shrimp; growth performances; immunity; water quality

INTRODUCTION

Shrimp culture is the most popular species for export-oriented aquaculture, which dominates the global seafood market. Though the shrimp industry has seen a great increase in market value over the last decade, shrimp diseases are still a major problem in the healthy development of this industry. Disease outbreaks have resulted in a decline in *Penaeus monodon* cultivation and severe losses in shrimp production. A current disease of farmed Penaeid shrimp, "Acute Hepatopancreatic Necrosis Disease" (AHPND), was first reported in southern China in 2010 and in Vietnam, Thailand (2011), and Malaysia (2010). The AHPND disease affects shrimp post-larvae within 20–30 days after stocking and causes up to 100% mortality (Tang et al., 2020). AHPND has caused a significant impact on shrimp production; losses accounted for nearly 60% of total losses and resulted in USD 43 billion across Asia (China, Malaysia, Thailand, and Vietnam) (Shinn et al., 2018; Kumar et al., 2021). In Malaysia, AHPND was first reported in mid–2010 in the peninsular states of Pahang and Johor, on the country's east coast. The disease spread to the states of Perak, Pahang, Penang, and Kedah in 2011 (Kua et al., 2016). Clinical signs of AHPND-infected shrimps include a pale-to-white and atrophied hepatopancreas, an empty stomach and midgut, the appearance of black spots or streaks visible within the hepatopancreas (due to melanized tubules), and soft shells at the chronic phase of the disease (Tang et al., 2020).

Immunostimulants are chemicals, drugs, stressors, or actions that facilitate defence mechanisms or the immune response, thereby improving an animal's innate defence mechanisms and providing protection against pathogens (Anita & Khati, 2019). Immunostimulants derived from natural plant products, such as medicinal plants, have been documented as part of current research in aquaculture (Trejo-Flores et al., 2018; Fierro-Coronado et al., 2019; Kaleo et al., 2019; Saptiani et al., 2020). In addition, natural plant products are reported to possess medicinal properties such as anti-stress, growth promotion, appetite stimulation, tonic, immunostimulation, and aphrodisiac and antimicrobial properties in finfish and shrimp larviculture, which are linked to the presence of active components such as alkaloids, flavonoids, pigments, phenolics, terpenoids, steroids, and essential oils (Esther & Ekundayo, 2022).

Piper betle is a perennial plant in the *Piperaceae* family with silky, heart-shaped leaves. *Piper betle* has also been reported to possess a good antibacterial agent against pathogenic microorganisms. The leaf extract showed antibacterial efficacy against several Vibrio species (Dayang Nur Jazlyn & Kian, 2017). Meanwhile, (Ataguba et al., 2018) revealed that the utilization of *Piper betle* extract at high doses demonstrated better inhibition activity in vitro and in vivo against several types of fish pathogens, thus suggesting its potential as a feed additive to prevent disease infection. To date, there have been limited studies on the utilization of *Piper betle* extract as an immunostimulant, mainly in tiger shrimp (*Penaeus monodon*) farming. However, little research has been done to highlight the medicinal plant's potential properties, particularly in improving the growth performance and immunity level of Tiger shrimp. A study by (Ataguba et al., 2018) indicated that during in vivo assessment through feeding of Nile tilapia (*Oreochromis niloticus*) with the ethanol extract of *Piper betle,* the number of white blood cells (WBC) was enhanced and significantly improved the rate of survival in the infected fish following challenge with *Streptococcus agalactiae.*

Therefore, the present study was conducted to investigate the potential properties of *Piper betle* extracts at various dosages on water quality parameters (pH, salinity, ammonia, nitrite, and nitrate levels), growth performances, and immunity level of tiger shrimp (*Penaeus monodon*) through a bath immersion approach. The methanolic extract of *Piper betle* at different concentrations of 0.00 mg/L (control), 0.05 mg/L, 0.10 mg/L, and 0.15 mg/L was incorporated into the experimental tank of cultivated Tiger shrimp for the duration of the experiment.

METHODOLOGY

Preparation of plant extract

The fresh *Piper betle* leaves were collected and cleaned with tap water to remove any contaminants and dirt. Next, the leaves were dried out at room temperature for a few days and further dried in an oven for 48 hours at 40°C. The dried leaves were ground into powder by using a Waring blender. The methanol extraction of the *Piper betle* was conducted following the method described by (Syahidah et al., 2014). Fifty grams of *Piper betle* in powder form were soaked in 250 ml of 80% methanol at a ratio of 1:5 sample to solvent. The sample was incubated on a rotary shaker for 72 hours. The extract was vacuum filtered and concentrated at a low temperature of 40°C in the rotary vacuum evaporator. The extract was dried in an oven at 40°C. The final extract was stored at 4°C in an airtight bottle.

Cultivation of tiger shrimp

The tiger shrimps were maintained in lab conditions and fed twice daily with the same commercial feed at a rate of 6% of their body weight for 3 weeks. The experimental conditions were maintained according to the standard parameters: dissolved oxygen (DO > 5), pH between 7.0 to 7.5, and temperature between 28.0 to 32.0 °C. Different concentrations of *Piper betle* extract were incorporated into the experimental tank and labelled as follows: 0 mg/L (control), 0.05 mg/L, 0.10 mg/L, and 0.15 mg/L. All tanks were maintained under continuous aeration throughout the period of the experiment.

Analysis of water quality

Water quality for each treatment was monitored on a daily basis based on parameters including pH, salinity, and content of ammonia, nitrate, and nitrite. The salinity was measured using a refractometer (Atago), while the pH, ammonia, nitrite, and nitrate were measured using the saltwater master kit. Five ml of water from each tank was taken using the test tubes provided in the saltwater master kit, and the test was conducted following the protocol described by the manufacturer.

Growth performances analysis

The growth performance was recorded based on the average body weight and length gain. Total length was measured from the tip of the rostrum to the tip of the telson. The weight gain (WG), length gain (LG), and specific growth rate (SGR) (%) are observed and recorded every five days. All the calculations are as follows:

Body weight gain (BWG) = $Wt - Wi$
Body length gain (LWG) = $Lt - Li$
Specific growth rate (SGR) = $[(\ln Wt - \ln Wi)/T] \times 100$
(Notes: Wt – mean final weight, Wi– mean initial weight, Lt – mean final length, Li – mean initial length, T – feeding trial period in days)

Total haemocyte count

The analysis was conducted according to (Arizo et al., 2016) with slight modifications. The haemolymph (0.3 ml) was collected from each shrimp sample and diluted with 10 µl of a 0.4% trypan blue solution. The total number of viable cells was determined using a Neubauer hemocytometer chamber (Qiujing MC), which was observed under a light microscope (Olympus CX21). The viable cells in the four corner squares were then counted, and the total cells per mL were determined. The analysis was conducted every five days.

Total bacterial count

The effect of *Piper betle* extract on the growth of pathogenic bacteria from *Vibrio spp.* in the hepatopancreas, gut, and exoskeleton of control and treated shrimps was evaluated by the Total Viable Count (TVC) method, according to (Sankar et al., (2011). After surface sterilization of the collected shrimp, the entire hepatopancreas, gut, and exoskeleton were aseptically dissected and homogenised using a mortar and pestle and sterile saline. The resultant aliquot was serially diluted up to a 105 dilution and plated on TCBS (thiosulfate citrate bile salts sucrose agar) agar (Merck). The formation of colonies was observed after 18 hours of incubation at 37.0 °C.

RESULTS AND DISCUSSION

Analysis of water quality

The water quality was measured based on the level of pH, salinity, and content of ammonia, nitrate and nitrite level following immersion treatment of methanolic extract of *Piper betle* at different concentrations; 0 mg/L (control), 0.05 mg/L, 0.10 mg/L and 0.15 mg/L and shown in Figure 1.

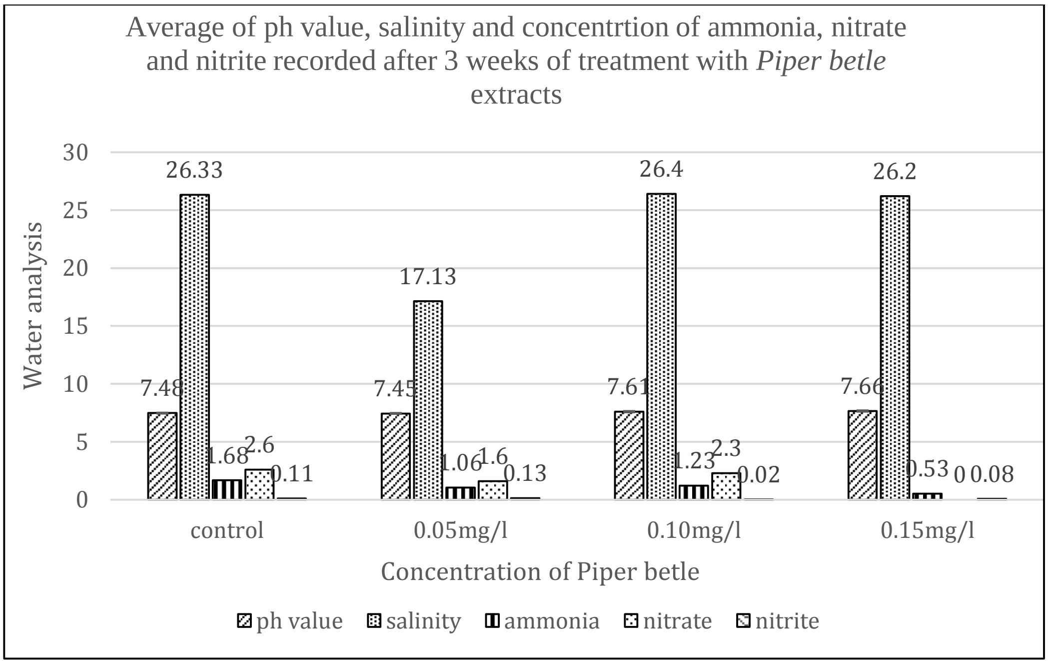

Figure 1 Average pH, salinity, ammonia, nitrate and nitrite level following treatment with 0.00, 0.05, 0.10, and 0.15 mg/L of methanolic extract of *Piper betle* into water tank of cultivated Tiger shrimp.

Analysis of the pH values from all the water tanks after treatment with 0.00 mg/L (control), 0.05 mg/L, 0.10 mg/L, and 0.15 mg/L of methanolic extract of *Piper betle* demonstrated an acceptable pH ranging between 7.48–7.66. According to Reddy and Mounika (2018), the optimal pH value for vannamei shrimp culture is 7.5–8.5, with a fluctuation range of 0.5. The mortality rate, however, is higher at high (> 8.5) and low (6.0) pH stresses (Han et al., 2018).

The salinity level of water treated with 0.00 mg/L (control), 0.10 mg/L, and 0.15 mg/L concentrations of *Piper betle* showed a value above 26 ppt, while water treated with 0.05 mg/L of *Piper betle* extract demonstrated a value below 17 ppt. The incorporation of a methanolic extract of *Piper betle* in water at a high concentration (between 0.10 mg/L – 0.15 mg/L) allows the salinity to be maintained at the optimum level. This dosage seems to be favourable for maintaining the salinity at an optimum level due to the shrimp's ability to naturally cultivate between 0 and 40 ppt as shrimp possess an osmotic haemolymph metabolism system (Esparza-Leal et al., 2018).

Analysis of ammonia levels shows that treatment with the highest concentration of *Piper betle* extract (0.15 mg/L) demonstrated the lowest value of ammonia concentration, followed by 0.05 mg/L and 0.10 mg/L. The control group, on the other hand, had the highest ammonia level when compared to the water treated with Piper betle extracts. The nitrate level in water treated with the highest concentration of *Piper betle* (0.15 mg/L) showed the lowest value of nitrate compared to other treatments (0.00, 0.05, and 0.10 mg/L). The analysis of the nitrite level showed that the water treated with 0.10 mg/L of *Piper betle* extract demonstrated the lowest value of the nitrite level compared to the control and other treatments.

This showed that the addition of *Piper betle* extract at various concentrations in the artificial seawater had influenced the levels of ammonia, nitrate, and nitrite. The higher levels of ammonia, nitrate, and nitrite can deteriorate water quality, and the long exposure to these toxicants alone or in combination can significantly attenuate the animal host's immunity, thus resulting in an increased risk of infections by pathogens such as *Enterocytozoon hepatopenaei* (EHP) (Nkuba et al., 2021) and White Spot Syndrome Virus (WSSV) (Kathyayani et al., 2019). This discovery emphasized the significance of maintaining optimal mean water quality parameters and minimizing fluctuation, which lowers the risk of disease and stress occurrence.

(Aaqillah-Amr et al., 2021) reviewed the evidence that water quality directly affects feeding responses in decapods, which rely on their chemical senses for foraging and social interactions; thus, low water quality may result in a low feeding rate. Several studies reported that the utilization of immunostimulants, particularly those derived from medicinal plants, has gained attention due to their potential to improve aquaculture production, especially in maintaining water quality during the cultivation period. A study by (Rao et al., (2017) reported that the application of the commercial herbal feed supplement Phytozoi at 3 g/kg feed improved water quality, increased survival rates, increased feed intake, promoted proper moulting, and also improved the hepatopancreas of the *Litopaneaus vannamei* in the treatment ponds (P2 and P3) compared to the control ponds (P1 and P4).

Analysis of growth performances

Tiger shrimp that were exposed to a 0.15 mg/L concentration of *Piper betle* demonstrated the highest weight (g) and length (cm) gains compared with the control (0.00 mg/L) and other treatment groups (0.05 mg/L and 0.10 mg/L). The tiger shrimp's specific growth rate (SGR) was significantly different ($P<0.05$), with values of -1.91%, 2.87%, and 1.21% for treatments of 0.00 mg/L (control), 0.05 mg/L, and 0.10 mg/L, respectively. The highest concentration of *Piper betle* extract, 0.15 mg/L demonstrated the highest SGR value, which is 5.87% in comparison with other treatments, while the control group demonstrated a negative SGR value.

This finding indicated that bath immersion treatment through the addition of a 0.15 mg/L concentration of *Piper betle* into the cultivation water was able to enhance the growth performance of the cultured tiger shrimp. Increased secretion of digestive enzymes, which facilitates the breakdown of food materials and the availability of nutrients for absorption, may also be attributed to improved growth performance (Rao et al., 2020). These could explain the improvements in weight gain, length gain, and SGR values in tiger shrimp that received the treatment with *Piper betle* extract compared to the control shrimp.

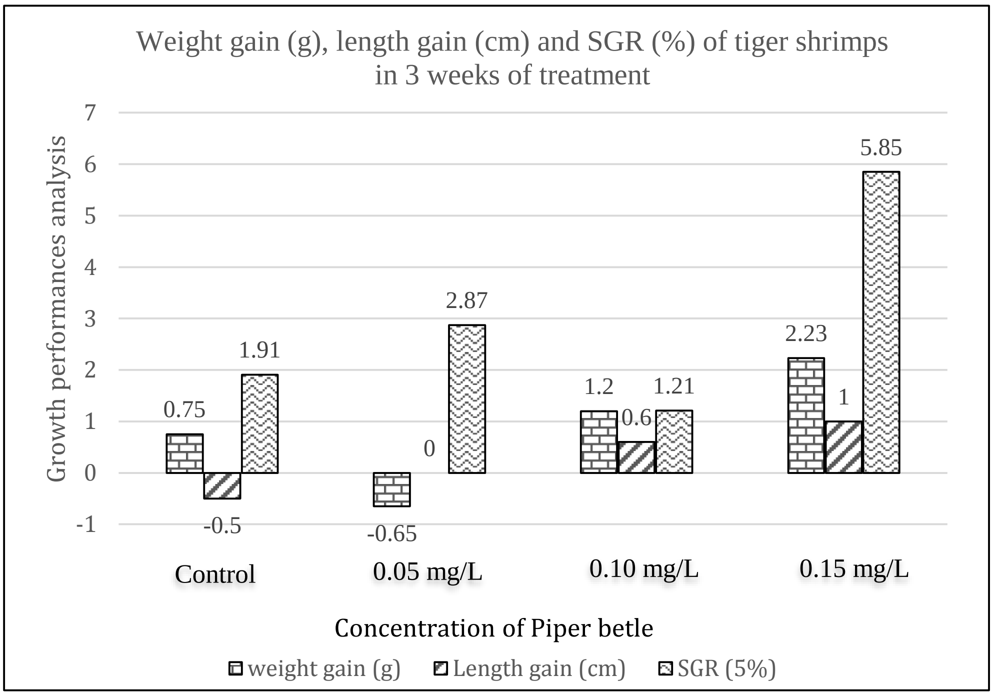

Figure 2 Weight gain (g), length gain (cm) and specific growth rate (SGR) of Tiger shrimp following treatment with 0.00, 0.05, 0.10, and 0.15 mg/L of methanolic extract of *Piper betle* into water tank of cultivated Tiger shrimp.

Analysis of immunity level

The immunity level of treated tiger shrimp was evaluated based on the analysis of total haemocyte count. During the first week of analysis, the haemocyte count was recorded as Mean ± SD (x10^7 cells/ml), which are $1.85^a \pm 1.16$, $5.71^a \pm 5.0$, $2.93^a \pm 2.7$ and $2.23^a \pm 1.24$ for treatment with 0.00 mg/L (control), 0.05 mg/L, 0.10 mg/L, and 0.15 mg/L of methanolic extract of *Piper betle* respectively. By the end of the experiment, tiger shrimp that exposed with the highest concentration of *Piper betle* at 0.15 mg/L demonstrated the highest number of haemocyte value which is $3.01^c \pm 0.04$ (x10^7 cells/ml) and the lowest value was recorded in the control group of $1.47^a \pm 0.04$ (x10^7 cells/ml).

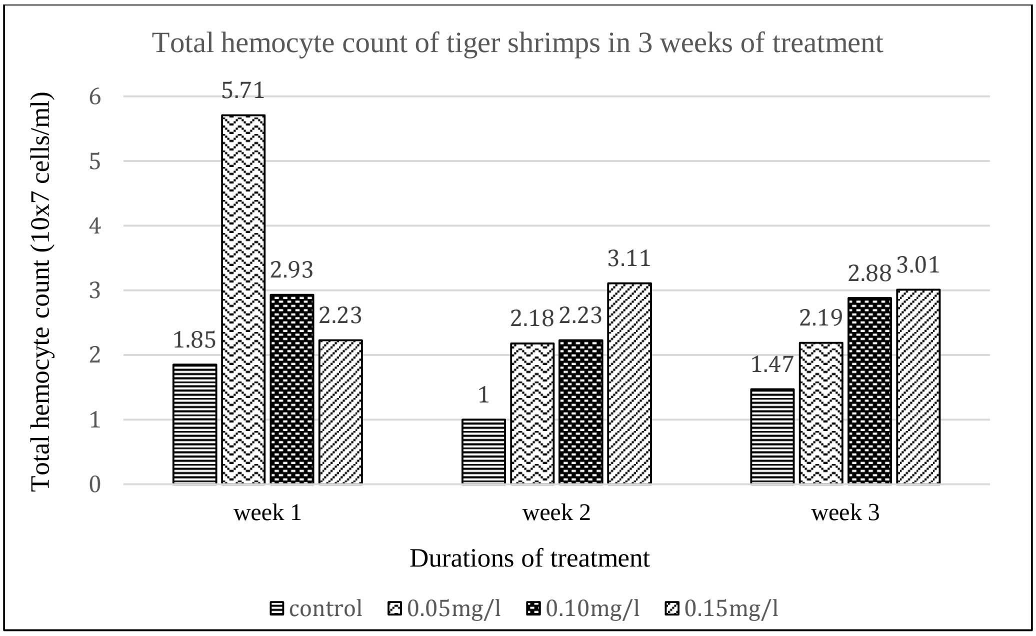

Figure 3 Total hemocytes count of Tiger shrimp following treatment with 0.00, 0.05, 0.10, and 0.15 mg/L of methanolic extract of *Piper betle* into water tank of cultivated Tiger shrimp.

In the present study, a significant increase in haemocytes count was demonstrated in the treatment group of 0.15 mg/L of *Piper betle* methanolic extract at week 2 and week 3. This may be due to an increase in cellular response, which represents the innate immune response of tiger shrimp. There were several studies reported on the modulation and improvement of immunity levels in shrimp after treatment with immunostimulants.(Arizo et al., 2016) reported that the immersion of *Machrobrachium rosenbergii* in 1% *Gracilaria edulis* before and after infection exhibited a significantly improved total haemocyte count and phenol oxidase levels 7 days post-infection with the white spot syndrome virus (WSSV). Meanwhile, (Saptiani et al., 2020) investigated the potential effect of *Xylocarpus granatum* leaf extract immersed in *Penaeus monodon* culture, that was effectively inhibited *Vibrio harveyi* infection, increased survival rate, and haemocytes cell of the *Peneaus monodon*.

Analysis of total bacterial load

Analysis of the total bacterial count indicated that the tiger shrimp samples treated with 0.15 mg/L of *Piper betle* extract had the lowest bacterial count compared to the other treatments (0.00 mg/L, 0.05 mg/L, and 0.10 mg/L). On the other hand, the control samples (0.00 mg/L) showed the highest number of bacterial counts, notably isolated from the exoskeleton and hepatopancreas. The reduction in bacterial count observed in the exoskeleton, gut, and hepatopancreas of treated tiger shrimps treated with *Piper betle* extract may be due to the potential antibacterial activity of *Piper betle* which inhibits the growth of bacteria. Based on the analysis, it shows that the application of *Piper betle* extract at a higher concentration may help to reduce the number of pathogenic bacteria such as *Vibrio* spp. Some Vibrio species and strains are pathogenic and infectious, leading to the disease "vibriosis" (de Souza Valente & Wan, 2021).

In another study using different medicinal plants, (Wachid et al., 2022) discussed the effectiveness of Meniran leaf extract (*Phyllanthus niruri* L.) as an immunostimulant in *Litopenaeus vannamei* against Vibriosis disease. The study found that there is a significant effect ($P< 0.05$) on the total shrimp gut bacteria, total viable count (TVC), and total bacterial count (TBC) isolated from the intestine of experimental shrimps. A study by Abang Zamhari and Kian (2017) reported that the methanolic extract of *Piper betle* extract exhibited the strongest antibacterial activities against varieties of *Vibrio* spp. The antibacterial activities using the disc diffusion test indicated that *Piper betle* extract showed good inhibitory activity with the diameter observed at 14 mm to 17 mm against the tested shrimp pathogens (*Vibrio harveyii*, *Vibrio alginolyticus* and *Vibrio parahaemolyticus*). Meanwhile, MIC and MBC values of *Piper betle* leaves reached 0.39 and 0.39 mg mL-1, respectively (Harlina et al., 2022). Therefore, the reduction in bacterial count in tiger shrimp could be due to the potential antibacterial activity demonstrated by the methanolic extract of *Piper betle*.

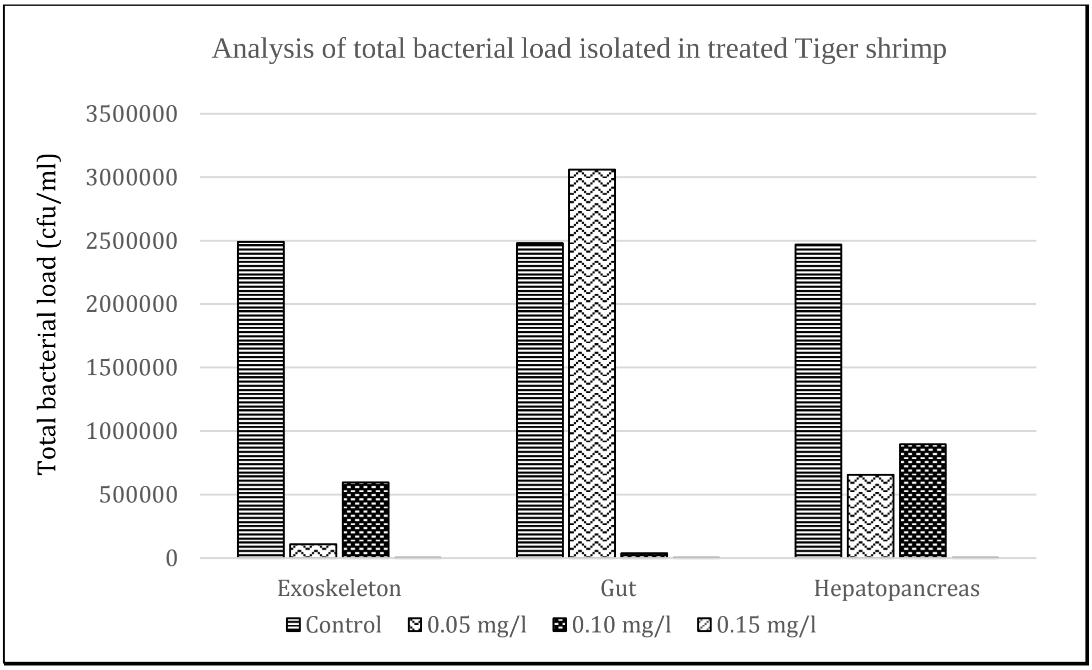

Figure 4 Total bacterial load of Tiger shrimp following treatment with 0.00, 0.05, 0.10, and 0.15 mg/L of methanolic extract of *Piper betle* into water tank of cultivated Tiger shrimp.

CONCLUSION

Tiger shrimp that were treated with the *Piper betle* methanolic extract through a bath immersion approach demonstrated better growth performance and immunity level based on weight gain (g), length gain (cm), specific growth rate, SGR (%), total haemocytes count ($x10^7$ cells/ml) and total bacterial load (cfu/ml). The *Piper betle* methanolic extract at 0.15 mg/L can be considered as the optimum concentration, which is sufficient to improve water quality, increase the growth performance, and enhance the immunity level in tiger shrimp.

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