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Research Article

Effects of dietary administration of Nettle leaves (*Urtica dioica*) hot water extract on health indices and immunity in *Litopenaeus vannamei* exposed to *Vibrio harveyi*

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Abstract

This study investigated the effects of *Urtica dioica* (Nettle) on health indices, including total hemocyte count (THC) and differential hemocyte count (DHC), total plasma protein (TPP), and glucose level of hemolymph when the *Litopenaeus vannamei* was fed with diets containing different hot water extract concentrations of *U. dioica* (10, 50, and 100 mg kg^{-1}) for 40 days. After 14 days of exposure to *Vibrio harveyi*, the survival rate was determined. The results showed that the use of *U. dioica* extract led to a significant increase in the health indices, (THC and DHC). The significant increase ($p \leq 0.05$) was observed in the frequency of small and large granular hemocyte, as well as in the biochemical indices, the number of hyaline cells, TPP, and glucose level, particularly at higher concentrations of the extract. After 14 days, the survival rate of the shrimps fed with a diet containing 50 mg kg^{-1} of *U. dioica* extract, showed a significant difference from other experimental groups. Overall, the hot water extract of the *U. dioica* can improve health indicators, THC, TPP, and glucose levels, and also increase the survival rate of the shrimp infected with *V. harveyi*.

Keywords: Whiteleg shrimp, Immunity, Survival rate, Medicinal herb, Vibriosis

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Introduction

Aquaculture has emerged as a solution to fulfill the protein demands of an expanding global population, owing to depleted fish stocks and escalating demand. In recent decades, the aquaculture industry has been significantly affected by viral and bacterial diseases, especially white spot viral disease and vibriosis. Chemical drugs and antibiotics are toxic and can cause drug resistance in pathogenic microorganisms. Thus, their use is restricted or banned in many countries. In contrast, probiotics and herbal compounds are commonly added to aquaculture feeds and they can prevent and control diseases of aquatic animals. In this regard, probiotics and phytomedicines are appropriate to use in aquaculture nutrition because of their safety, low toxicity, and minimal environmental impacts. According to the research, herbal medicine has an essential role in enhancing the immune functions of aquatic animals and effectively increases antiviral, antibacterial, and antiparasitic activities of the immune functions (Ghaednia *et al.*, 2011; De Vico *et al*., 2018; Bababaalian Amiri *et al.*, 2020; Forouzani *et al.*, 2021). Currently, many researchersare study on the immunological role of individual herbs extracts, allowing precision medication and reducing development costs (Zhu, 2020).

The medicinal plant, *Urtica dioica* (commonly known as Nettle), is a herbaceous perennial plant that grows up to a height of 8-10 cm and features trichomes with hooks or cones (Golalipour and Khori, 2007), belongs to the Urticaceae family (Bnouham *et al.*, 2003) and has been recognized as a traditional medicinal plant, widely employed for treating various ailments across different regions of the world. *U. dioica* is traditionally used as an herbal medicine in Western Asia. This plant is traditionally used in Morocco, Turkey, Brazil, Jordan, and with much frequency in Northern Iran (Hosseinkhani *et al.*, 2017). *U. dioica* is abundant in humid areas of Iran, especially in the northern, western, and central regions of the country, in provinces such as Golestan, Mazandaran, Azerbaijan, Lorestan, Khuzestan, Fars, Kashan, Bushehr and Isfahan reported (Buso *et al.*, 2020). Among the six species within this plant genus, *U. dioica* and *U. uren*s have been particularly regarded for their medicinal properties for a long duration (Motamedi *et al.*, 2014). *U. dioica* was employed in the management of various diseases, such as angina, hemoptysis, cancer, and spleen diseases (Bhusal *et al.*, 2022). Notably, *U. dioica* is highly effective in managing blood sugar in the host and comprises 20 various bioactive compounds like neophytadiene accounting for 25.21% of total compounds, in addition to several aromatic compounds such as carboxylic acid (7.63%) and fatty acid ester compounds. Moreover, different parts (*e.g.* leaves, flower, and rhizome) and forms (*e.g.* crude, extract, and active ingredient) of *U. dioica* are used to modulate specific biological functions (*e.g.*, growth promoter,

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immunostimulants or immunomodulators, appetite stimulation, antibacterial, and anti-virus) (Tadese *et al.*, 2022) even in fish and shrimp (Modarresi-Chahardehi *et al.*, 2012; Zare *et al.*, 2023). Effect of *U. dioica*, as a medicinal plant feed additive, has been evaluated in the fish with the improved growth performance, proximate body composition (Zare *et al.*, 2021), immune system, and resistance against infectious pathogens (Awad *et al.*, 2013; Mehrabi *et al.*, 2020). The literature also briefly pointed out the positive dietary effect of nettle on fish growth performance and the immune system (De Vico *et al*., 2018). Some of these immunostimulatory effects of *U. dioica* extracts, were reported by various researchers (Golalipour and Khori, 2007; Samakar *et al.*, 2022).

Equilibrium between aquatic animals and microorganisms may break sometimes due to irresponsible or inadequate handling, and sometimes it may lead to diseases (Karunasagar *et al*., 1994) and can cause high mortality. Vibriosis is one of the most common bacterial disease problems in the aquaculture centers of shrimp farms, and on several occasions, it has caused massive mortalities (Karunasagar *et al*., 1994; Vandenberghe *et al.*, 1998). Diseases with bacterial etiology, particularly *Vibrio* species, have inflicted loss on the shrimp farming industry worldwide, especially in Asian countries (Jory and Ngo, 2014) as well as south of Iran. Vibriosis is considered a secondary infection that usually proliferates when cultured shrimp has become immunologically weak (Moriarty, 1998; Rezaee *et al.*, 2020). According to the reports, *V. harveyi* continues to cause chronic mortalities up to 30% of shrimp larvae, post larvae, and adults under stressful conditions. *Vibrio* species cause infection at all life stages (from eggs to broodstock); generating in most cases 100 % mortality (Prayitno and Latchford, 1995; Harris and Owens, 1999). Some *Vibrio* species identified to cause vibriosis include *V. harveyi*, *V. vulnificus*, *V. parahemolyticus*, *V. alginolyticus*, *V. penaeicida* (Brock, 1990; Ishimaru, *et al.*, 1995). Two types of *Vibrio* infections, namely acute hepatopancreatic necrosis disease (AHPND) and systemic vibriosis, are more commonly observed in shrimp farms (Kumar *et al.*, 2020).

In this research, the effects of different dietary levels of *Urtica dioica* L. (Nettle) water extract on hemolymph health indices, total hemocyte count (THC), differential hemocyte count (DHC), total plasma protein (TPP), glucose level and survival rate after exposure to *V. harveyi* in *Litopenaeus vannamei* were studied.

Materials and methods

Hot water extract of Urtica dioica

The fresh leaves *of U. dioica* were collected from Salmanshahr city, located in the northern Mazandaran province of Iran. Subsequently, the leaves were dried at laboratory temperature with suitable ventilation, ground, and sieved out by using a 400µ mesh, to prepare a fine powder (Harhaun *et al.*, 2020). This step was crucial to achieve an appropriate balance between the solvent

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and solute components (Gharekhani *et al.*, 2010). Then, 60 g of the powder was extracted by the Soxhlet Apparatus (Singh *et al.*, 2013) using 250 mL solvent (deionized water) for 90 min (Pan *et al.*, 2002) and filtrated with Whatman filter paper No. 42 before proceeding to the final stages of concentration and drying. The extract was dried using an oven (Memmert model UNB 400), and the specific weight of the resulting extract was calculated and transferred to the suitable container. The container was sealed with parafilm and maintained in the refrigerator at 4°C until daily usage (Merchie *et al.*, 1998).

Diet preparation

For the preparation of the experimental diets, a commercial feed (38% crude protein, 9% fat, 3% fiber, 14% ash, and 10% moisture) was provided from the Havoorash feed factory (Iran, Bushehr). The commercial feed was powdered and mixed in a mixing machine. Then, the cornstarch and water were added (Mirbakhsh *et al.*, 2022). The hot water extract of *U. Dioica* was weighed according to 10 (T1), 50 (T2), and 100 (T3) mg kg^{-1}, added to sterile distilled water, and mixed well with the powdered ingredients until the formation of paste. Subsequently, the experimental diets were pelleted using a grinder machine, and each treatment was placed separately in an oven (60°C and 6 h). These diets were then packaged and kept in a freezer (−20°C). All steps were performed in the control group without adding the hot water extract of the *U. dioica* (Tsai *et al.*, 2019; Ghosh *et al.*, 2021). The feed for each tank was prepared according to the biomass per tank. The feeding was initiated twice a day at 9 a.m. and 5 p.m.

Experimental design

The shrimps were transferred from the Helle site to the Iran Shrimp Research Center, in Bushehr province, south of Iran. One hundred and twenty juveniles *L. vannamei* with (4.08±1.20 g) were randomly selected. The shrimps were transferred to the challenge laboratory in 300 L tanks. The shrimps were allowed to acclimate for 7 days before the experiment (FAO, 2003). During the experiment, the tanks had the same photoperiod. Water exchange was carried out up to 20% of each tank every day and wastes were removed.

Five experiments were conducted in triplicates (30 shrimps per tank). The negative and positive control groups (NC and PC) were fed with a commercial diet and three experimental groups were fed with supplemented diets with 10, 50, and 100 mg kg^{-1} hot water extract of the *U. dioica* named T1, T2, and T3 groups, respectively. The average weight of shrimp post-larvae was 4.06±0.05 g at the initial stocking time. According to the average weight of the under-trial shrimp, they were fed for 40 days at 5% of the shrimp weight (Zhang *et al.*, 2021).

Throughout the experiment, the physical and chemical parameters of the water were monitored daily using a digital multi-parameter instrument (HACH model HQ40d multi). The

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parameters analyzed included temperature, pH, dissolved oxygen, and salinity, which were determined using a visual gauge (JENCONS ATAGO S/mill-E). The salinity, temperature, and dissolved oxygen were measured 30-32 ppt, 29-31ºC, and 7 ppm, respectively. The pH ranged from 7.2 to 7.5.

The shrimps were selected randomly in each treatment, and the hemolymph samples were collected from the abdominal sinus area by sterile syringes (Vargas-Albores *et al.*, 1993). The sterile syringes with 1mm needles contained 0.4 mL of Alsever solution as an anticoagulant (Na Citrate 27mM, NaCl 333mM, glucose 115mM, EDTA 9mM, pH=7) (Jiang *et al.*, 2004; Ghaednia *et al.*, 2011). The hemolymph samples were collected and transferred to small sterile Eppendorf tubes for centrifugation. The total hemocyte count (THC), differential hemocyte count (DHC), and total plasma protein (TPP) were measured from the collected samples.

Total hemocyte count (THC)

The number of total hemocytes was determined by a hemocytometer. Twenty-five microliters of aspirated hemolymph of each shrimp were placed on the slide using a Marienfeld model Neubauer-improved bright-line Hemocytometer. The slide was observed under an optical microscope (Nikon model Eclipse E200, Japan) with a 40× magnification and the hemocyte cells were counted.

Differential hemocyte count (DHC)

To determine the DHC, hemolymph smears were prepared on glass slides with three replications for each hemolymph sample and then stained by the May-Grunwald-Giemsa staining method (Ghaednia *et al.*, 2012) following which an optical microscope with a magnification of 100× was used to count 100 cells. Based on the size and shape of the nucleus relative to the cytoplasm, the Hemocytes were classified into three types, which were Hyaline, Small Granular, and Large Granular cells (Van De Braak, 2002).

Biochemical markers total plasma protein (TPP) and glucose

The remaining hemolymph for each sample in the Eppendorf tubes was centrifuged and used to measure the total plasma protein by the Biuret method (Acharya *et al.*, 2004). The glucose concentration was determined using the enzyme glucose oxidase GOD-PAP method (Rascón-Careaga *et al.*, 2021) in a diagnostic laboratory.

Exposure of treated L. vannami to V. harveyi

After 40 days, the challenge was carried out by the injection method. *V. harveyi* IS01 (PTCC 1759) was grown on Tryptic soy agar medium with 2.5% NaCl for 18h at 30°C (Mirbakhsh *et al.*, 2014). After incubation, cells were harvested by centrifugation at 4750×g for 10 min, washed, and suspended in sterile normal saline to adjust the number of bacteria at 10^4–10^5 CFU/mL)

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(Tang *et al.*, 2013). The prepared bacteria solutions were injected into the ventral sinus of the cephalothorax to achieve doses of 20 µg per shrimp (Huang *et al.*, 2013). Shrimps in all treatments except NC were exposed to *V. harveyi*. After infection, the accumulated mortality of the shrimp was recorded for 14 days.

Statistical analysis

The results were analyzed by One-way ANOVA. Duncan post-hoc tests were used to determine differences (p<0.05) between testing groups. All statistics were performed with Predictive Analytics Software (PASW), version 22 (IBM® SPSS®, USA). Three replications were carried out for all samples to ensure the accuracy and reliability of the results.

Results

Health Indices

After the shrimps were fed with diets containing different levels of hot water extract of *U. dioica* for 40 days, the THC in all treatments (T1, T2, and T3) showed a significant difference in the control groups (NC and PC) (p≤0.05). In addition, all treatments showed a significant difference with each other, and the highest amount of THC was observed in T2 (196.00 ± 2.59 ×10^5 cell mL^{-1}) and the lowest in treatment T3 (140.25±2.12 ×10^5 cell mL^{-1}) (Fig. 1A).

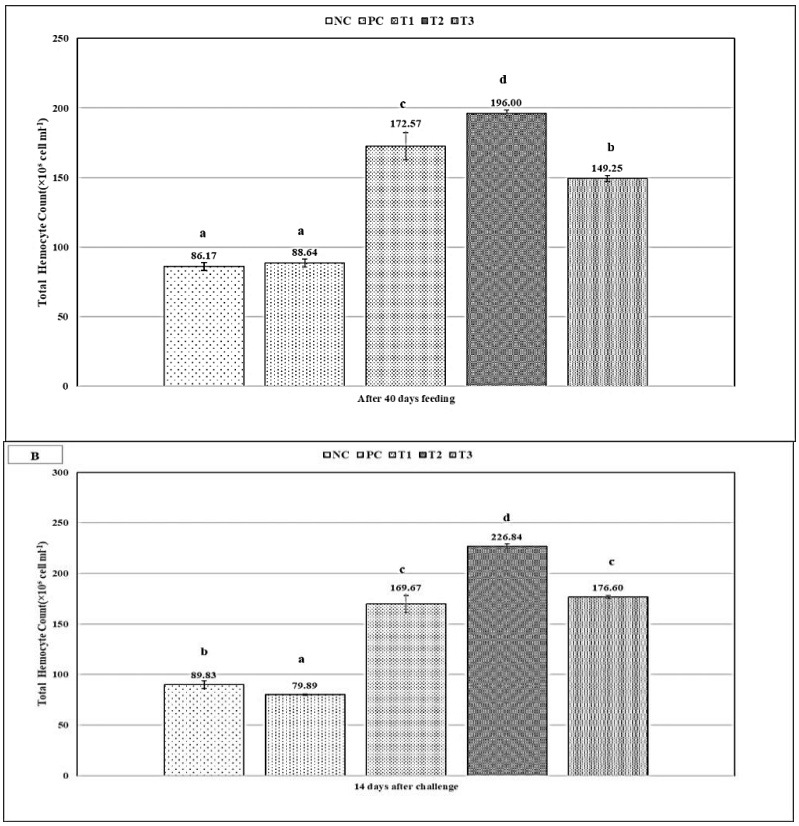
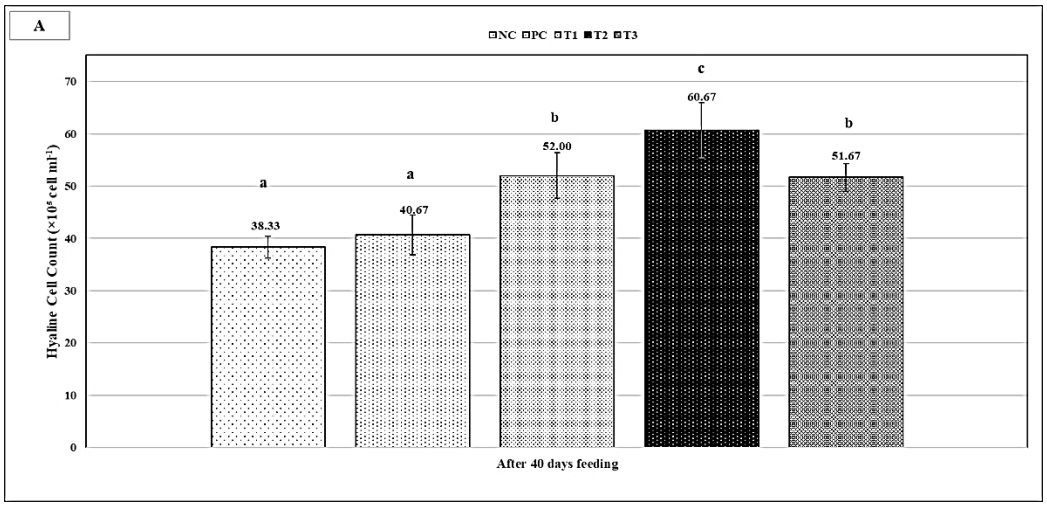


Figure 1: Total hemocyte count (THC) (×10^5 cell mL^{-1}) in the hemolymph of *Litopenaeus vannamei* **fed with diets supplemented with different levels of hot water extracts of the** *Urtica dioica* **after 40 days of feeding (A) and 14 days after exposure to** *Vibrio harveyi* **(B). Same superscript letters, indicate homogeneous subsets as determined by Duncan's test and are not significantly different (p≥0.05).**

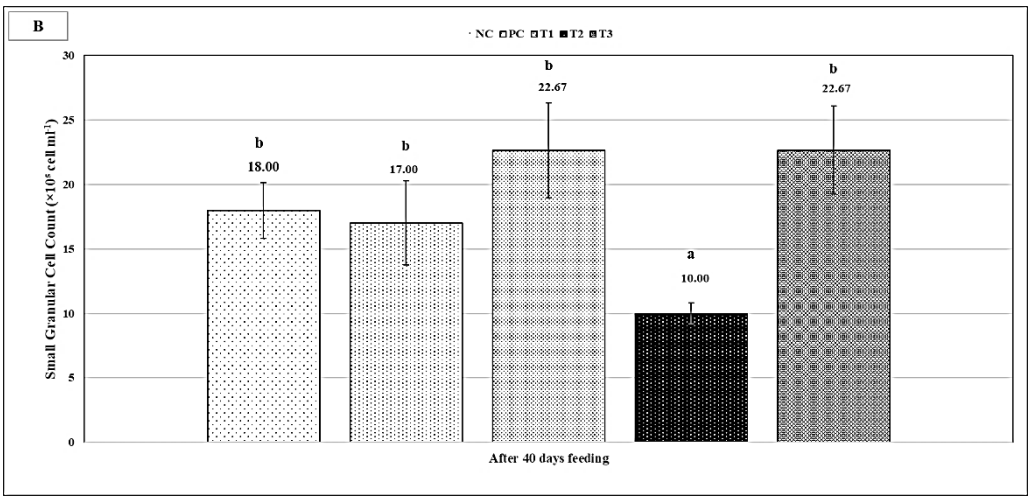
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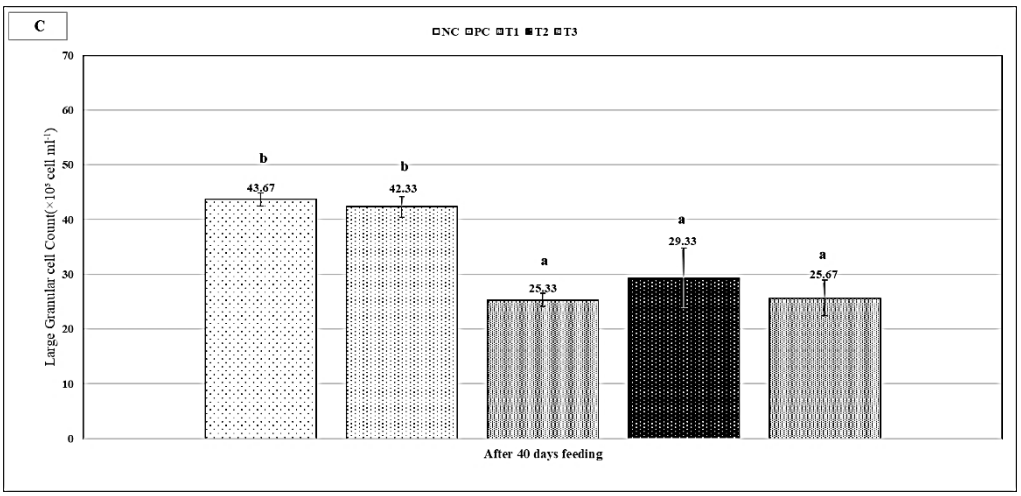
Fourteen days after shrimp exposure to *V.harveyi*, the frequency of THC showed a significant difference between the two control groups. The highest THC was observed in T2 ($226.84\pm2.45\times0^5$ cell mL^{-1}), and T1 and T3 showed no significant difference (Fig. 2A).

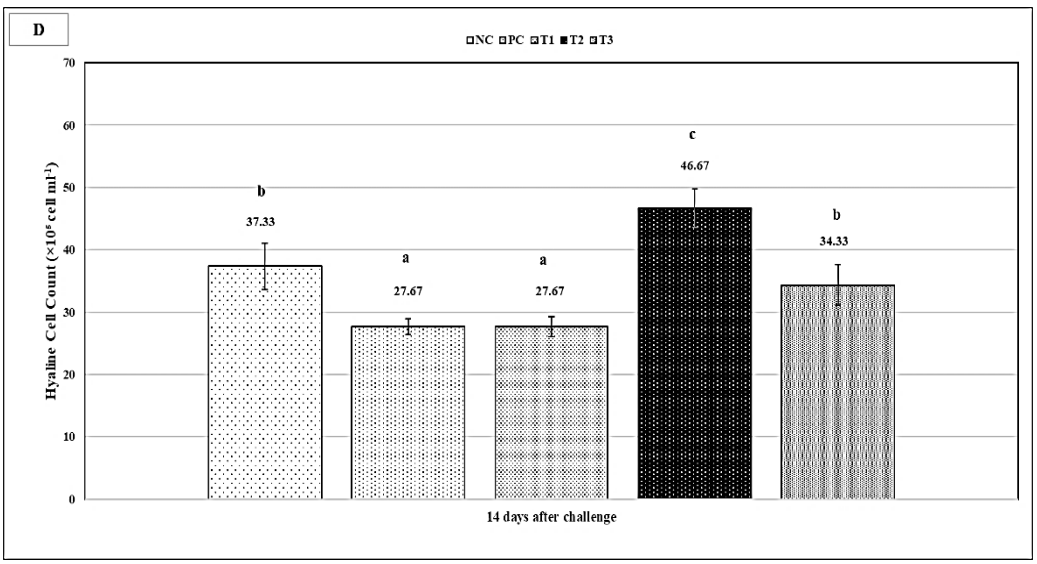
DHC was calculated based on the presence of hyaline, small granular, and large granular cells. At the end of the 40th day of feeding the shrimps in the treatments, the highest frequency of hyaline (H) cells was observed in treatment 2 ($60.67\pm5.25\times10^5$ cell mL^{-1}), and treatments 1 and 3 did not have significant differences (Fig. 2A). At the same time, the frequency of small granular (SG) cells was the lowest in treatment T2 ($10.00\pm0.82\times10^5$ cell mL^{-1}) ($p\leq0.05$), and no significant difference was observed between treatments T1 and T3 (Fig. 2B) but large granular (LG) cells frequency at the end of the 40th day of feeding was the same in all treatments and did not show any significant difference ($p\geq0.05$) (Fig. 2C). Fourteen days after shrimp exposure to *V.harveyi*, the highest frequency of H-cells was again in T2 ($46.67\pm3.09\times10^5$ cell mL^{-1}) was observed, and there was a decrease in the H cells frequency in the positive control. Although a significant difference between T2 and T3 ($34.33\pm2.04\times0^5$ cell mL^{-1}) was observed, there was no difference between T1 ($27.67\pm2.49\times0^5$ cell mL^{-1}) and PC ($p\geq0.05$). It should be considered that the frequency of H cells in NC ($37.33\pm3.68\times0^5$ cell mL^{-1}) and T3 ($10.00\pm0.82\times10^5$ cell mL^{-1}) had no significant difference ($p\geq0.05$). (Fig. 2D). The SG cell frequency between T1 and T3 and also between controls (NC and PC) and T2 ($16.67\pm1.70\times10^5$ cell mL^{-1}) showed no significant difference (Fig. 2E). The lowest and highest frequencies of LG cells were observed in the T2 and T1, respectively. The frequency of LG cells in T1 ($60.33\pm2.49\times10^5$ cell mL^{-1}) and T3 ($56.00\pm3.27\times10^5$ cell mL^{-1}) had no significant difference ($p\geq0.05$) (Fig. 2F).



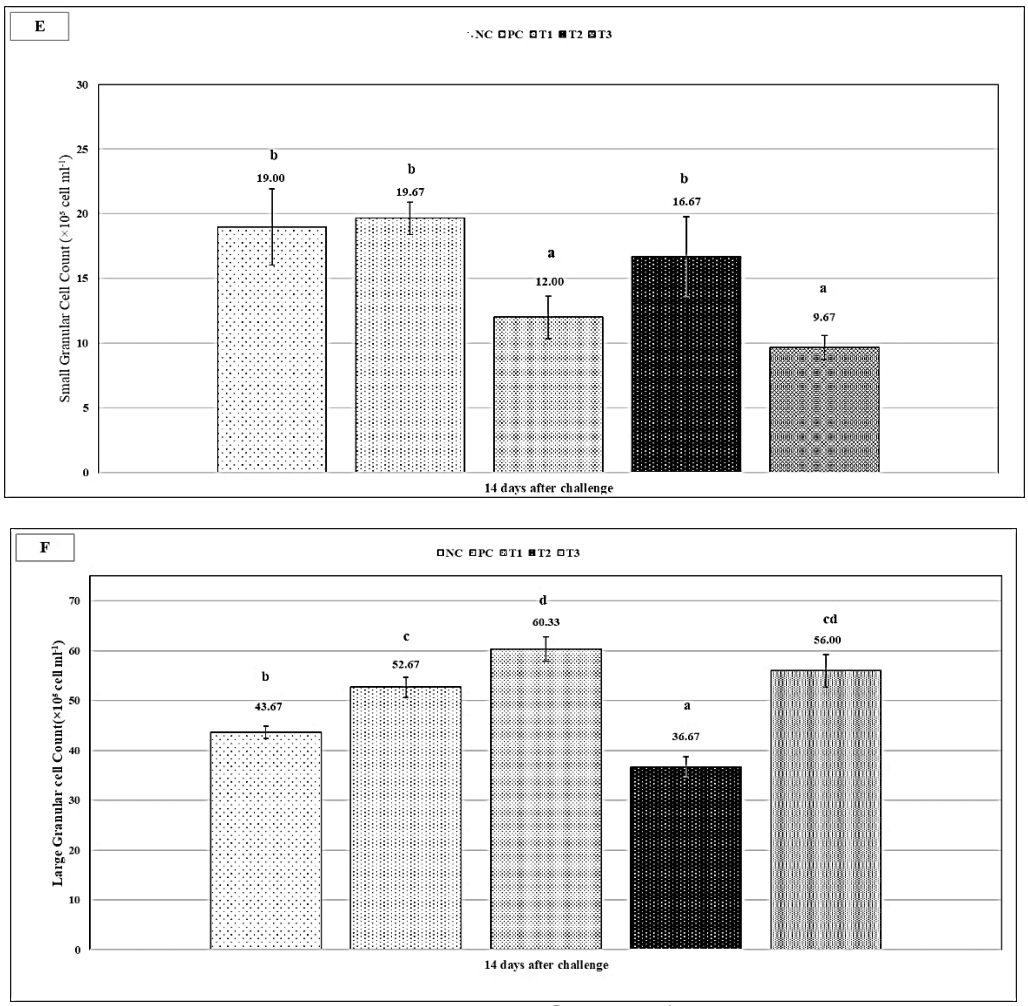
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Figures 2: Differential hemocyte count (DHC) (×10^5 cell mL^{-1}) in the hemolymph of *Litopenaeus vannamei* **fed with diets supplemented with different levels of hot water extracts of the** *Urtica dioica* **after 40 days of feeding (A, B, C) and 14 days after exposure to** *Vibrio harveyi* **(D, E, F). Same superscript letters, indicate homogeneous subsets as determined by Duncan's test and are not significantly different (p≥0.05).**

Biochemical indices of hemolymph

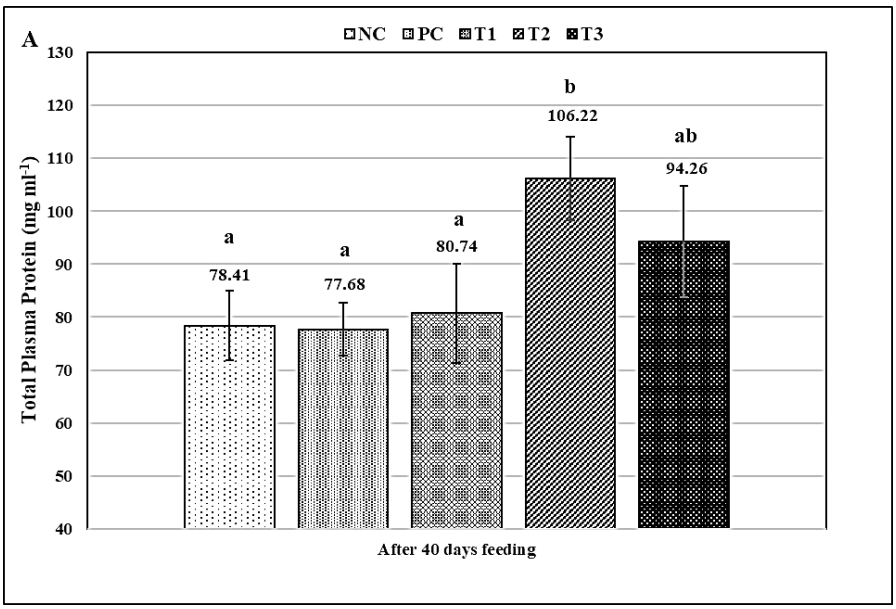
After 40 days of shrimps feeding with diets containing different levels of hot water extract of *U. dioica*, no significant difference was observed between TPP values in the control groups (NC and PC) and T1 (80.74±9.38 mg mL^{-1}) and T3 (94.26±10.48 mg mL^{-1}) (p≥0.05). The highest value of TPP was recorded in treatment T2 (106.22±7.88 mg mL^{-1}), which, although there was no difference between T2 and T3, was significantly higher than T1 (p≤0.05) (Fig. 3A). The glucose level did not show any significant difference between the control groups (NC and PC) and T1 (16.58±5.47 mg mL^{-1}), as well as between T2 (25.07±6.31 mg mL^{-1}) and T3 (29.80±7.30 mg mL^{-1}) (Fig. 3C). Fourteen days after exposure to *V.harveyi*, the values of TPP between NC (80.71±6.93 mg mL^{-1}) and PC (67.14±6.54 mg mL^{-1}) and also between T2 (111.92±7.44 mg mL^{-1}) and T3 (105.68±2.91 mg mL^{-1}) did not show significant differences (Fig. 3B). 14 days after exposure, although the glucose level of hemolymph in T1, T2, and T3

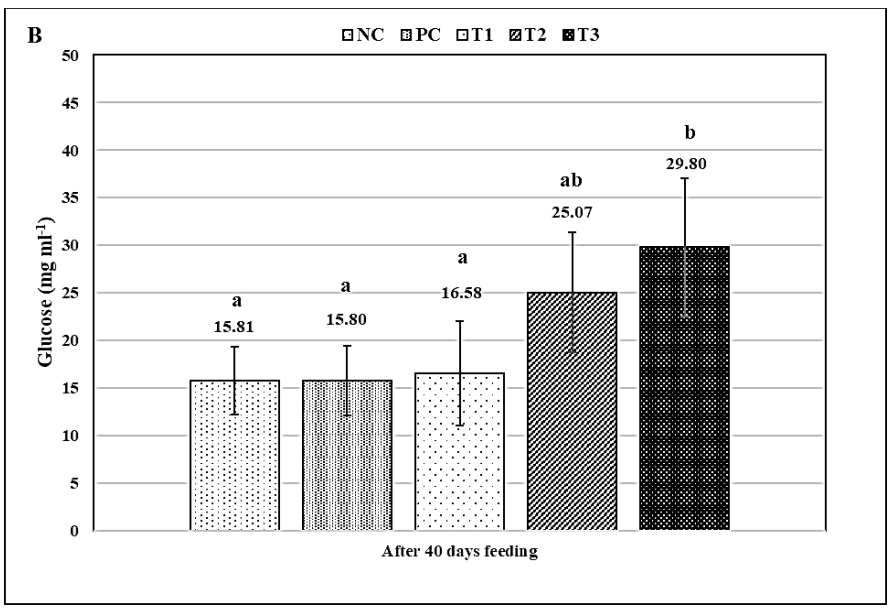
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did not show a significant difference, a significant difference was observed between NC (17.12±2.67 mg mL^{-1}) and PC (7.78± 1.08 mg mL^{-1}) (Fig. 3D).

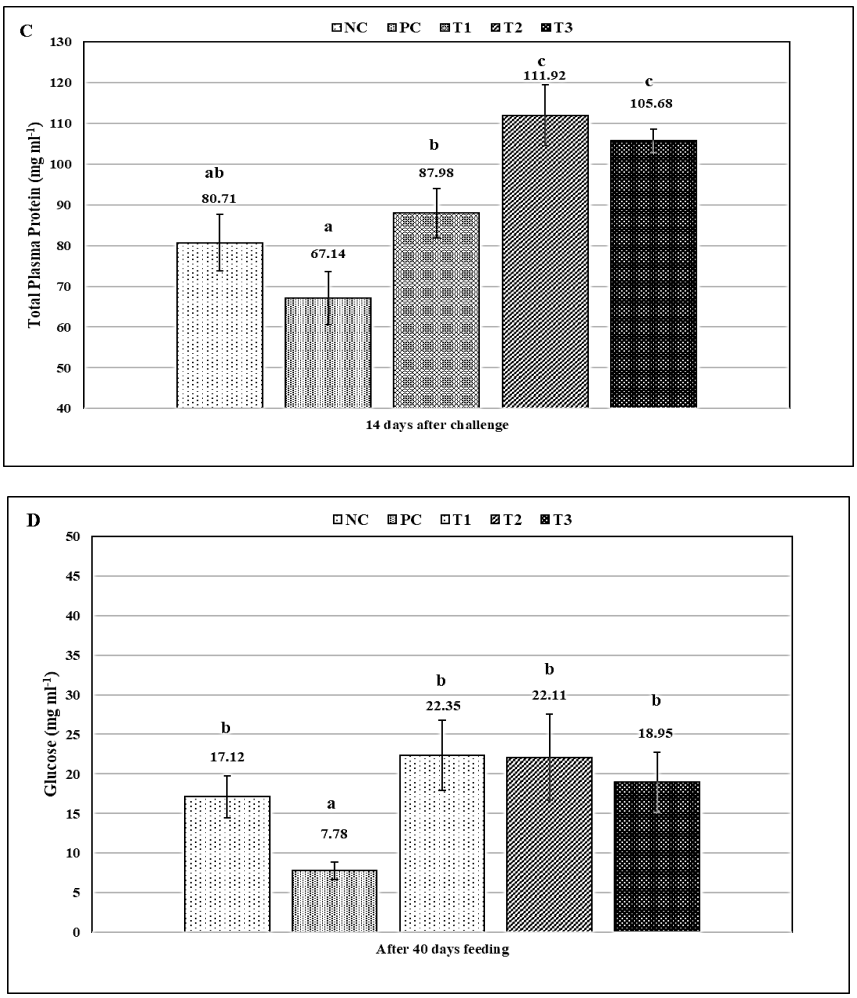
Survival rate after exposure to V. Harvey

The survival rate in the PC was 50.17±1.31 % at the end of 14th day of exposure to *V. harveyi*, while in the NC was 100 %. The low survival rate was recorded in the PC and the highest in T1 (91.67±2.87 %) and T2 (89.33±2.05 %) (Fig. 4).





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Figures 3: Biochemical indices of hemolymph (TPP and Glucose level) of *Litopenaeus vannamei* **fed with diets supplemented with different levels of hot water extracts of** *Urtica dioica* **after 40 days of feeding (A, B) and 14 days after exposure to** *Vibrio harveyi* **(C, D). Same superscript letters, indicate homogeneous subsets as determined by Duncan's test and are not significantly different ($p \geq 0.05$).**

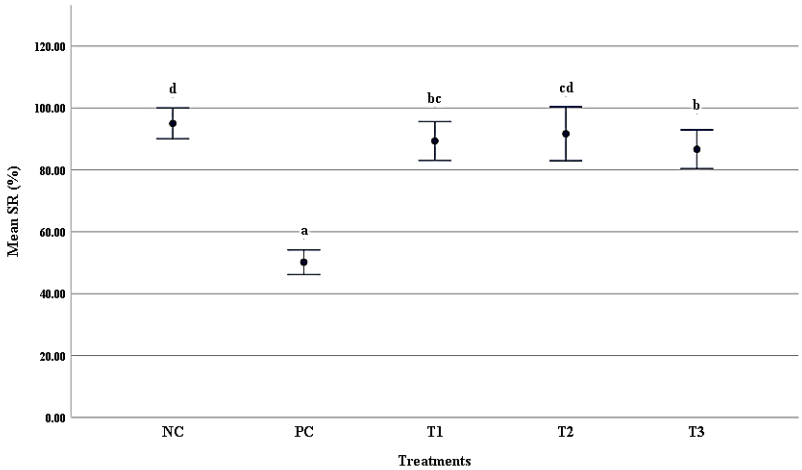


Figure 4: The survival rate of *Litopenaeus vannamei* **after 40 days of feeding with diets supplemented with different levels of hot water extracts of the** *Urtica dioica* **and 14 days after exposure to** *Vibrio harveyi* **(20 µl, 1.5×10^5 CFU mL^{-1}). Same superscript letters, indicate homogeneous subsets as determined by Duncan's test and are not significantly different ($p \geq 0.05$).**

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Discussion

Several advances have been made in aquaculture to enhance nonspecific defenses and develop resistance to common diseases. Aquatic animals are immersed in a suspension of microorganisms (Schulze *et al*., 2006). Therefore, the administration of immunostimulants is important because when overstimulated, physiological force is used to enhance the immune system rather than survival (Li and Xiang, 2013).

After the shrimp were fed diets containing different amounts of *U. dioica* hot water extract for 40 days, THC levels in T1, T2, and T3 showed a significant difference from the control groups (NC and PC), with the highest and lowest THC levels observed in T2 and T3, respectively. On the other hand, the increase in H cells in T2 and the increase in SG cells in T1 and T3 indicated that the shrimp may be resistant to bacterial or viral infections by adjusting the amount of hot water extract from *U. dioica* in the *L. vanammei* diet. Each hemocyte type has an individual role in the shrimp immune system. H cells are responsible for phagocytosis activity and nodule formation. LG cells are very active in cytotoxicity and activation of the prophenoloxidase (proPO) system, while encapsulation and activation of the proPO system are performed by SG cells (Bachère, 2000; Pope *et al.*, 2011).

The increase in TPP levels can be attributed to the effects of immune system stimulants on protein levels, followed by the production of antimicrobial peptides. In crustaceans, such peptides are produced as hemocyanin (Su *et al.*, 2022). The significant increase in TPP in T2 also confirms that the addition of 50 mg kg^{-1} of *U. dioica* hot water extract to the diet should result in an increase in the resistance of *L. vannamei* to bacterial infection. This conclusion is confirmed by recording the highest survival rate in T2 after 14 days of exposure to *V. harveyi*.

At the end of the 40th day of feeding, the number of SG cells increased in T1 and T3, showing a significant difference from T2. Considering the role of SG cells in encapsulating and activating the ProPO system, which is responsible for recognizing the invaders and encapsulating them (Van De Braak, 2002). Hemolytic nodules are formed by numerous hemocytes that act synergistically to capture microorganisms or large antigens that cannot be removed by phagocytosis (Johansson and Soderhall, 1989). These nodules are subject to subsequent activation of the proPO system, melanization, and microbial destruction (Pope *et al.*, 2011). Therefore, it is possible to explain the higher survival rate in T1 and T3 compared to NC and PC.

Increasing the number of LG hemocytes after 40 days of feeding shrimp in T1, T2 and T3 has been shown to increase the ability of the shrimp immune system to encapsulate, activate the proPO system and cytotoxicity, making the host more resistant to viral, bacterial, and parasitic diseases

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(Johansson and Soderhall, 1989; Bachère, 2000). H cells readily attach to foreign particles and are capable of phagocytosis, encapsulation, and nodulation (Rodrıguez and Le Moullac, 2000).

After shrimps exposed to *V. harveyi*, the highest level of THC and TPP in T2 confirmed that the diet containing 50 mg kg^{-1} hot water extract of *U. dioica* can stimulate the immune system. Rodrıguez and Le Moullac (2000) first used the term health indices for THC and TPP. The simultaneous increase of THC and TPP in T2 showed that the health indices were improved. The highest numbers of H cells in T2 showed that the shrimp had a greater capacity for phagocytosis. This conclusion is confirmed by the high survival rate in T2. This study were in agreement with Sudaryono *et al.* (2018).

The significant increase in LG cells in T1 and T3 shows that the 10 and 100 mg kg^{-1} hot water extract of *U. dioica* in the diet lead to an increase in the ability of the shrimp immune system in the synthesis, storage and secretion of the ProPO system, thus increasing the ability of the immune system to eliminate viral pathogens (Amparyup *et al.*, 2013).

Glucose level of hemolymph is an index that can be influenced by environmental conditions, feed, metabolic rates, and molting processes. In addition, these index may represent physiological responses to environmental and biotic factors such as stress, salinity, temperature, and the effects of diet in crustaceans (Rosas *et al*., 2000; Su *et al.*, 2022), as well as pathogenic bacteria such as *Vibrio* species, *Vibrio alginolyticus*, and resistance to them (Liu *et al.*, 2005). The extract of *U. dioica* has been used since ancient times as an agent for blood glucose control in humans. The studies on the effect of *U. dioica* on aquatic animals showed that the extract or powder of this plant either increased glucose levels (Saeidi Asl *et al*., 2017) or had no effect (Binaii *et al*., 2022). In the present study, the highest levels of glucose were measured in T2 and T3, However on the 14th day after exposure to *V. harveyi*, all treatments did not show significant difference with the NC, although there was a significant decrease in The PC was recorded. Similar findings in other aquatic animals are also different from the research results conducted on mice and humans (Dar *et al*., 2013).

In conclusion, feeding *L. vannamei* with a diet containing 50 mg kg^{-1} hot water extract of *U. dioica* leaves for 40 days stimulated the immune system, improved health indices (THC, DHC, and TPP), and increased survival rate after exposure to *V. harveyi*. Administration of 50 mg kg^{-1} hot water extract of *U. dioica* increased H cells but did no affect hemolymph glucose levels, whereas feeding a diet containing 100 mg kg^{-1} increased SG and LG cells and glucose levels.

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