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Research Article

Dietary ethanolic extract of *Prosopis juliflora* improved immune-antioxidant capacity and resistance to cold water stress in Pacific white shrimp, *Litopenaeus vannamei*

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Abstract

Prosopis species, particularly *Prosopis juliflora* are known for their antioxidant, anti-inflammatory, antibacterial, and antifungal properties. The present study evaluated the effect of three doses of dietary ethanolic leaf extract of *P. juliflora* (ELEPJ), i.e., 0.25, 0.5, and 1% on the immune-antioxidant system and the resistance to cold water stress in Pacific white shrimp, *Litopenaeus vannamei*. After 60 days of the feeding trial, the shrimps fed diets containing 0.5 and 1% ELEPJ showed significantly higher lysozyme activity, superoxide dismutase, phenol oxidase, and glutathione peroxidase than those of the control group (p<0.05). In response to the cold water stress, the shrimp fed diets containing 0.5 and 1% of ELEPJ showed significantly higher stress resistance than the control group. These results suggest the positive effects of ELEPJ on the immune-antioxidant system and stress resistance of *L. vannamei* and supplementation of 0.5-1% ELEPJ is recommended for the farm-raised shrimp diet.

Introduction

The Pacific white shrimp, *Litopenaeus vannamei*, stands as the predominant species in global aquaculture due to its rapid growth, adaptability to varying salinities, resilience against diseases, and environmental robustness (Chang *et al*., 2013; Sudaryono *et al.*, 2015; Purnamasari *et al.*, 2017). The utilization of natural products to augment the performance of farmed shrimp represents an advantageous strategy in the health management of shrimp aquaculture (Duan *et al.*, 2017). Incorporating safe and natural feed additives like medicinal herbs as dietary supplements has shown potential in enhancing feed efficiency, promoting growth, and fortifying the immune system of cultured shrimp. These eco-friendly and cost-effective approaches hold promise for bolstering production yields and averting disease outbreaks within shrimp aquaculture (Ali *et al*., 2008; Goda, 2008; Chen *et al*., 2019). Numerous herbs and plants, along with their derivatives, are recognized reservoirs of multifunctional curative agents and bioactive compounds (Deng *et al.*, 2012; Saini *et al.*, 2016; Akbary and Aminikhoei, 2018; Chirawithayaboon *et al.*, 2020).

Species of the genus *Prosopis*, particularly *Prosopis juliflora*, have emerged as some of the most aggressive invasive alien plant species worldwide, notably in semi-arid and arid regions. They were introduced to various parts of the world, including Africa, India, Australia, and the Middle East, to serve diverse purposes such as providing shade, shelter, fodder, fuelwood, timber, and enhancing soil stability in degraded ecosystems (Almaraz-Abarca *et al.*, 2007; Shiferaw *et al.*, 2021). The leaves of *P. juliflora* exhibit noteworthy antioxidant activity attributed to the presence of alkaloids, tannins, flavonoids, coumarins, and anthraquinone glycosides. Moreover, they contain a diverse array of secondary metabolites, conferring unique and multifaceted medicinal properties (Ahmad *et al.*, 1989; Rastogi and Mehrotra, 1993; Badri *et al*., 2017; Saleh and Abu-Dieyeh, 2021). Various extracts derived from *P. juliflora* have demonstrated a broad spectrum of biological activities, including antioxidant, antihyperglycemic, antibacterial, anthelmintic, antitumor, and anticancer properties (Zhong *et al.*, 2022). The well-established antioxidant potential of the ethanolic leaf extract of *P. juliflora* (ELEPJ), attributable to its rich composition of alkaloids, tannins, flavonoids, coumarins, and anthraquinone glycosides, further underscores its significance (Saleh and Abu-Dieyeh, 2021).

Few studies have delved into the impact of dietary *P. juliflora* on aquaculture species. Notably, Chovatiya *et al*. (2018) found that incorporating *P. juliflora* pods and beans into the diet of rohu fingerlings, *Labeo rohita* led to improved growth performance and higher protein content in the fish's carcass. Similarly, de Souza *et al.*, 2021) observed no adverse effects on the health or survival of Nile tilapia, *Oreochromis niloticus* when substituting corn meal with *P. Juliflora* meal. Despite the acknowledged antioxidant and immunostimulant properties of *P. Juliflora*, no prior studies have investigated its effects on the immune system and antioxidant

capacity of aquaculture species, particularly those with limited immune defenses, such as crustaceans.

Additionally, crustaceans are susceptible to low temperatures, which can induce physiological dysfunction, leading to compromised oxidative and immune defenses in shrimp (Qiu *et al*., 2011; Xu *et al*., 2018; Wang *et al*., 2019; Schleder *et al.*, 2020; Ren *et al.*, 2021; Xu *et al*., 2021; Yang *et al*., 2022). It has been documented that *L. vannamei* ceases feeding at temperatures below 18°C and faces mortality at temperatures under 12°C (Kumlu *et al*., 2010; Li *et al*., 2015). Such low water temperatures are prevalent in shrimp farms, especially during the winter season. In the realm of shrimp aquaculture, diminished temperatures can compromise shrimp health by impairing immune responses and disrupting normal physiological functions. Furthermore, a decline in temperature beyond this point leads to oxidative stress, overwhelming the antioxidant defense mechanisms, and results in inhibited immune function (Zhu *et al.,* 2024).

Therefore, this study aims to examine the impact of dietary ethanolic leaf extract of *P. Juliflora* (ELEPJ) on the immune-antioxidant system and resistance to cold water stress in Pacific white shrimp, *L. vannamei*.

Materials and methods

Diet preparing

To prepare the ELEPJ, we followed the method outlined by Saleh and Abu-Dieyeh (2021), with slight modifications. In summary, leaves of *P. Juliflora* were meticulously collected, thoroughly washed, dried, ground, and then stored at 4°C until the extract was ready for preparation. The *P. juliflora* leaf powder was soaked in 70% ethanol at a weight-to-volume ratio of 1:10 for two days at room temperature. Subsequently, the solution underwent filtration, and extraction was facilitated using a rotary evaporator. The resulting extract was then transferred to a glass Petri dish and placed under a laminar hood for evaporation and drying. The resulting ELEPJ was reconstituted in 100 mL of distilled water and subsequently sprayed onto the commercial shrimp feed sourced from Faradaneh, Iran. This feed was composed of various ingredients, including fish meal, soybean meal, shrimp powder, and rapeseed meal. The feed pellets measured 2.2 to 2.5 mm in diameter and 4 to 6 mm in length.

Experimental design

Six hundred shrimp, initially weighing 7.1±2.1 g, were divided into four groups: a control group receiving a diet without ELEPJ, and three experimental groups receiving diets with 0.25%, 0.5%, and 1% ELEPJ, respectively. The administrated doses were selected according to the previous studies in shrimp (Abidin *et al.* 2022; Baniesmaeili *et al.* 2023; Santhosh *et al*., 2023). Within each treatment, 150 shrimps were randomly distributed across 300 L circular fiberglass tanks, with three replicates (50 shrimps per tank). Following a two-week acclimation period in the 300 L tanks and subsequent feeding with commercial shrimp feed, the shrimp were transitioned to the experimental diets for 60 days. Water conditions, including temperature (30±2˚C), oxygen levels

(8.2±0.5 mg L^{-1}), pH (7.5), and salinity (35±0.47 g L^{-1}), were meticulously maintained. The study was conducted in strict adherence to the ethical guidelines outlined by the Iranian Society for The Prevention of Cruelty to Animals and the Canadian Council on Animal Care.

Immune-antioxidant parameters

After 60 days, hemolymph samples were extracted from three specimens per tank, following the established protocols detailed in Akbarzadeh *et al*. (2019) and Niroomand *et al.* (2020). Subsequently, the activities of lysozyme glutathione peroxidase (GPx), superoxide dismutase (SOD), and phenol oxidase (PO), were assessed. Lysozyme activity (U mL^{-1}) was assessed using the turbidimetric assay following Ellis's method (1990), with modifications and employing hen egg white lysozyme as a standard. A suspension of *Micrococcus luteus* (Sigma, USA) at a concentration of 0.2 mg mL^{-1} was introduced to the plasma, while PBS served as the negative control. Lysozyme activity was defined as a reduction in absorbance of 0.001 per minute, and one unit represented this reduction. Superoxide dismutase (SOD) activity (U mL^{-1}) was determined using a commercial kit (ZellBio GmbH, Germany) per the manufacturer's instructions. Under enzymatic reaction conditions, the superoxide anion was converted to hydrogen peroxide and oxygen, generating a specific chromogen leading to a measurable color change at 420 nm. Phenol oxidase (PO) activity was measured spectrophotometrically. The plasma samples were initially diluted in TBS-1 and pre-incubated with an equal volume of the enzyme inducer trypsin (Sigma) at 20˚C for 15 minutes. Subsequently, the trypsin-plasma combination was replaced with TBS-1. Upon addition of l-DOPA, DOPA-chrome formation was monitored at 0, 5, 15, and 25-minute intervals. PO activity was quantified as the change in absorbance (490 nm) per minute and expressed as U mL^{-1}. Glutathione peroxidase (GPx) activity (U mL^{-1}) was determined using a commercial kit (ZellBio GmbH, Germany), measuring the amount of sample necessary to catalyze the decomposition of 1 µmole of GSH to GSSG within one minute.

Low-temperature stress

At the end of the feeding period, shrimp were subjected to an acute cold water stressor. Ten shrimp were randomly selected from each tank and placed in 50 L containers. The water temperature was then gradually lowered to 7˚C, and the shrimp were exposed to this cold environment for 12 hours, following the protocol established by Eshagh Nimvari *et al*. (2019). Subsequently, the number of mortalities was meticulously recorded, and the survival rate was calculated using the formula:

Survival (%) = 100 × (initial shrimp number−dead shrimp number) / (initial shrimp number)

Statistical analysis

All data were presented as means±standard deviation (SD). Prior to statistical analysis, the normality of the data was assessed using the Shapiro-Wilk test. The dataset was then subjected to one-way ANOVA, followed by multiple comparisons utilizing Turkey's post-hoc test for finding significant effects

at a significance level of (p<0.05). All statistical analyses were conducted using the SPSS program (version 16), and the data were visualized using Sigma Plot (version 11).

Results

Table 1 presents the results, indicating significantly elevated levels of the activity of all studied antioxidant enzymes (GPx, SOD, and PO) as well as lysozyme activity in the shrimps fed diets containing 0.5% and 1% ELEPJ compared to the control group (p<0.05). Furthermore, the PO activity was notably higher in shrimp fed the diet containing 0.25% ELEPJ compared to the control group (p<0.05).

Table 1: The results of immune-antioxidant biomarkers of hemolymph of shrimp fed with 0.25, 0.5, and 1% leaf extract of *Prosopis juliflora* after 60 days of feeding trial.

Biomarkers	Treatments			
	Control	**0.25%**	**0.5%**	**1%**
Lysozyme activity (U mL^{-1})	4.61 ± 0.26^{c}	5.31 ± 0.34^{bc}	6.44 ± 0.99^{a}	5.98 ± 0.53^{ab}
Phenol oxidase activity (U mL^{-1})	2.48 ±0.32^{b}	3.36 ± 0.35^{a}	3.44 ± 0.29^{a}	3.41 ±0.76^{a}
Superoxide dismutase activity (U mL^{-1})	93.26 ± 2.97^{c}	95.96 ± 3.04^{bc}	105.40 ± 4.18^{a}	103.40 ± 9.85^{ab}
Glutathione peroxidase activity (U mL^{-1}l)	48.1 ± 1.61^{b}	49.56 ± 1.60^{b}	53 ± 1.41^{a}	52.75 ± 1.69^{a}

Data (mean ± SD) with different letters are significantly different among treatments according to the ANOVA and Turkey's post-hoc test (p<0.05).

The tolerance of experimental shrimp to cold water stress is illustrated in Figure 1. Shrimp fed with 0.5% and 1% ELEPJ exhibited significantly higher survival rates in response to cold water temperature stress compared to the control treatment (p<0.05). Additionally, shrimp fed a diet containing 0.25% ELEPJ demonstrated a significantly higher survival rate compared to the control group (p<0.05).

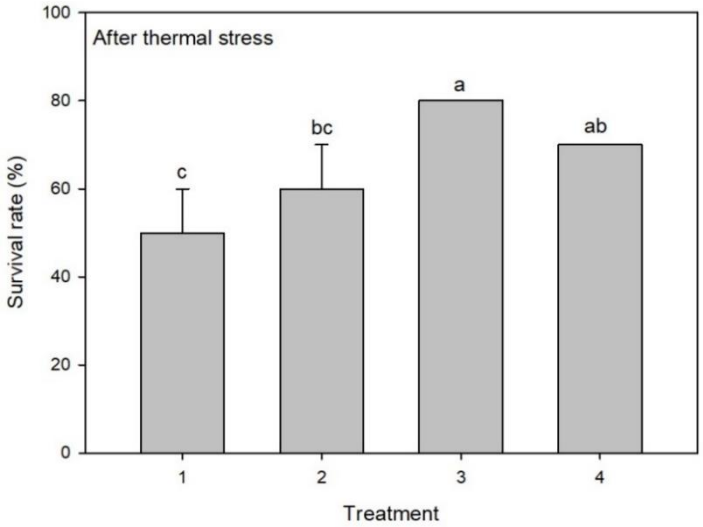

Figure 1: Survival rate of shrimp fed with 0.0 (1) 0.25 (2), 0.5 (3), and 1% (4) leaf extract of *Prosopis juliflora* after 60 days of feeding trial. Data (mean±SD) with different letters are significantly different among treatments according to the ANOVA and Turkey's post hoc test (p<0.05).

Discussion

Using the herbal extracts in aquafeeds is known as the least expensive and safe approach to stimulate the immune system in aquatic animals (Azizi *et al.*, 2016; Haghighi *et al.*, 2019; Van Doan *et al.*,

2020). Given the nutritional value of the *P. juliflora* leaf and its extensive availability as an aggressive invasive alien plant species worldwide, the present study examined for the first time the effectiveness of dietary ELEPJ on the antioxidant capacity and resistance to cold water stress in Pacific white shrimp, *L. vannamei*. The results showed that the hemolymph antioxidant enzymes and the resistance to cold water stress were remarkably improved in response to dietary ELEPJ in shrimp.

The immune-antioxidant capacity of serum serves as a reliable indicator of the overall health status of animals (Ma *et al*., 2022). In response to dietary ELEPJ, the immune-antioxidant capacity of *L. vannamei* exhibited a remarkable enhancement. Plasma levels of GPx, SOD, PO, and the activity of lysozyme enzyme demonstrated significant increases in the shrimps fed diets containing 0.5% and 1% ELEPJ. These antioxidant enzymes play a crucial role in safeguarding cells against oxidative stress (Hernández-Muñoz *et al.*, 2000; Downs *et al.*, 2002). The extract of *P. juliflora* is characterized by its potent antioxidant activity, owing to its abundance in secondary metabolites and natural antioxidants including flavonoids, alkaloids, tannins, phenolic compounds, and saponins (Ahmad *et al.*, 1989; Rastogi and Mehrotra, 1993; Badri *et al*., 2017; Saleh and Abu-Dieyeh, 2021). The ethanolic extract of *P. juliflora* has exhibited high concentrations of phenolic and flavonoid compounds, showcasing significant antioxidant and free radical scavenging potential (Saleh and Abu-Dieyeh, 2021). These enzymatic antioxidants possess the capability to mitigate the effects of oxygen free radicals and reactive oxygen species (ROS) in aerobic organisms, thus shielding cells from oxidative stress (Zhong *et al*., 2022). Recent studies have highlighted various herbal extracts, such as *Rubus coreanus* (Subramanian *et al*., 2013), Guava, *Psidium guajava* (Dewi *et al*., 2021), Moringa oleifera (Abidin *et al*., 2022; Baniesmaeili *et al*. 2023), Indian ginseng, *Withania somnifera* (Abdel-Tawwab *et al.*, 2022), and Ginkgo biloba (Liao *et al*., 2023), for their ability to enhance the immune-antioxidant capacity in shrimp.

Our findings demonstrate an enhanced tolerance to cold water stress in shrimp that were fed with *P. juliflora* leaf extract compared to the control treatment. This effect may be attributed to the stimulating influence of ELEPJ on the immune-antioxidant capacity of shrimp. Like other crustaceans, *L. vannamei* cannot regulate its body temperature and is particularly sensitive to low temperatures (Ren *et al.*, 2021). Cold stress is known to induce physiological dysfunction, including DNA damage, as well as impairments in oxidative and immune defenses in shrimp (Qiu *et al*., 2011; Xu *et al.*, 2018; Wang *et al*., 2019; Schleder *et al*., 2020; Yang *et al*., 2022). This stressor can prompt the generation of reactive oxygen species (ROS) and disrupt the balance of the antioxidant defense system in crustaceans (Xu *et al*., 2021). In response to cold stress, *L. vannamei* exhibits immunological impairment, an increase in apoptosis, and a reduction in ROS scavenger activity (Fan *et al*., 2013). While *L. vannamei* can elevate its enzymatic antioxidant defense system and non-specific immune activities to

mitigate oxidative stress caused by cold water stress, severe cold stress can lead to mortality in *L. vannamei* (Han *et al*., 2022; Wang *et al.*, 2023). Previous studies on *L. vannamei* have demonstrated that feeding with various levels of mangrove *Avicennia marina* leaf powder led to improved immunity indices and enhanced survival under cold temperature stress (Eshagh Nimvari *et al*., 2019). It is plausible that the heightened levels of immune-antioxidant enzymes in the shrimps fed diets containing ELEPJ contribute to an increased resistance to low-temperature stress.

Conclusions

This study marks the inaugural demonstration of the positive impacts of incorporating ELEPJ into the diet of *L. vannamei*, a crustacean species, on the immune-antioxidant system, ultimately leading to improved survival rates under low-temperature stress. The heightened resilience observed in ELEPJ-fed shrimp during cold stress could be attributed to the induced levels of immune-antioxidant enzymes. Based on our findings, it is recommended to include up to 1.0% ELEPJ in the diet of farmed *L. vannamei* for optimal results.

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Conflicts of interest

The authors declare that they have no conflict of interest.

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