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Assessing the immunomodulatory effect of ashwagandha (*Withania somnifera*) root powder in dietary supplementation on *Penaeus vannamei* against *Vibrio parahaemolyticus*

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Abstract

The present study was conducted to investigate the effect of ashwagandha (*Withania somnifera*) root powder on immunological parameters and disease resistance to *Vibrio parahaemolyticus* in *Penaeus vannamei* (Boone, 1931). The active metabolites withanolides containing ashwagandha root powder (ARP) formulated with a 35% protein level as a different dietary concentrations viz. T₀ (Control, 0%), T₁ (2.5%), T₂ (5%) and T₃ (7.5%). *Penaeus vannamei* post-larvae (average weight 0.012g) were selected with a density of 20 no. in each of the 16 treatment tank and distributed in completely randomized design. The ARP feed diets in *P. vannamei* exhibited significantly higher values in WG, SGR (%), and PER, and significantly lower values in FCR in the treatment T₃ feed diet as compared to the control diet (0% ARP), with a significant level of $p < 0.05$. The disease challenge test of *Vibrio parahaemolyticus* (0.1 ml injection) randomly selected shrimps in each tank and exhibited higher SR (%) in treatment T₃, with a significance level of $p < 0.05$. The ARP supplemented in feed diet significantly increased the immunomodulatory levels such as Prophenoloxidase activity (PPO), Respiratory burst of superoxide anion production (NBT assay), and the total haemocyte count in haemolymph in treatment T₃ feed diet with a significance level of $p < 0.05$. Thus, at the end of the result, the supplemented 7.5% of ARP in shrimp feed diet was interpreted without any adverse effects on growth performance and immune responses of *L. vannamei*.

Keywords: Withanolides, *Withania sonifera*, *Penaeus vannamei*, immune parameters, growth performance, disease resistance, *Vibrio parahaemolyticus*

Introduction

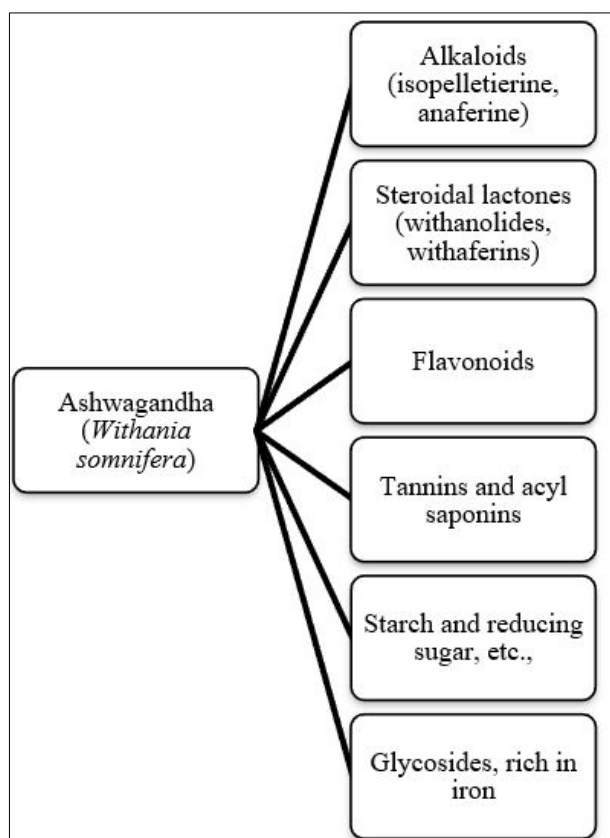
The world aquaculture is one of the rising sectors for the development of global food supply and food security needs and also the tool for the developing national economy. The white-leg shrimp (*Penaeus vannamei*) has superior traits and is the most popular cultivable species in Asian penaeid shrimps (Liao and Chein, 2011) [19]. The usage of antibiotics is gradually increasing in the aquaculture sector for the urgent needs of industries for their development. As the demand for food quality continues, the antibiotics used in aquaculture also increase; they can be replaced or modified by the aquatic animal cells genetically to create a severe impact on the health of humans and animals life (Quesada *et al.*, 2013) [28].

Vaccines and immunostimulants are the keys used for disease management in aquaculture which increase the immunity of the aquatic animals. Immunostimulants are drugs that enhance the immune systems more resistant to microbial infections (Dalmo, 1995) [9] and as dietary additives for the stimulation of defence mechanisms and improve resistance to specific pathogens (Lundén and Bylund, 2000) [21]. Immunostimulants provide complete protection against pathogens by triggering the host's immunological response to produce more antibodies. As many kinds of immunostimulants are available in the global market, they are in the forms of polysaccharides, hormones, vitamins, microorganisms, physiologically active materials, herbs, and synthetic medications which can improve the immune system and cause a strong defence response in the host (Sakai, 1999; Mohapatra *et*

al., 2013; Buchmann, 2014; Kasahara and Satoh, 2014; Song *et al.*, 2014; Shrestha *et al.*, 2015) [32, 24, 7, 14, 37, 34].

Several original feed additives primarily sourced from yeast and herbal extracts, known as "phytobiotics," control microflora by promoting a beneficial composition, encouraging helpful bacteria growth, and inhibiting potentially harmful microbes and parasites. In the opinion above, the Indian herbal plant Ashwagandha (*Withania somnifera*) belongs to the family Solanaceae and has been an important herb in Ayurvedic, Siddha and Unani medical systems for various physical disorders. Particularly the root of Ashwagandha called 'winter cherry' (Indian ginseng) has been selected for incorporation with feed formulation. Withanolides, an active component present in Ashwagandha promote health and are responsible for defence longevity against diseases.

Apart from the immunostimulatory response of Ashwagandha, also well known for its anti-inflammatory, anti-stress, antioxidant, adaptogen, antibiotic, aphrodisiac, astringent deobstruent, diuretic and sedative properties (Sivaram *et al.*, 2004; Mirjalili *et al.*, 2009) [35, 22]. Therefore the present study aims to investigate the immunomodulatory effect of Ashwagandha root powder in dietary feed supplementation (2.5%, 5%, 7.5%) on white leg shrimp, *Litopenaeus vannamei* (Boone, 1931) [5] against *Vibrio parahaemolyticus*.



Source: Mishra *et al.*, 2000; Mirjalili *et al.*, 2009; Mukherjee *et al.*, 2019) [23, 22, 10]

Fig 1: Biologically active compounds present in Ashwagandha (*Withania somnifera*)

Materials and methods

White-leg shrimp juveniles

A 60-day feeding experiment was conducted at College of Fisheries Science, Veraval. A post-larvae PL of *P. vannamei* (average initial weight, ± 0.012 g) was obtained from a

commercial hatchery near Veraval, Gujarat. Shrimps were acclimatized in an indoor rectangular FRP tank (500 L) for 7 days with continuous aeration before the feeding trial began at a temperature (28°–30°C); pH (7.3); dissolved oxygen (5 – 5.4 mg L⁻¹); alkalinity (121 – 127 mg of CaCO₃ L⁻¹); salinity (14 – 15.6 ppt). During this period, the cultured shrimps were fed twice in the morning and evening with a control diet containing 35% dietary protein level.

Experimental Design and setup

An experiment was conducted in rectangular plastic aquaria (60.1cm × 30.5cm × 30.5cm) filled with 45 L of filtered (100µm) seawater. To adjust the salinity, the seawater was diluted with freshwater. At the beginning of the trial experiment, the PLs of white-leg shrimp were carefully selected from the stocked FRP tank and directly distributed into 16 aquaria (4 replicates per treatment) with an initial stocking density of 20 PL/tank. Three experiment treatment levels of dietary supplementation of Ashwagandha root powder (2.5%, 5%, and 7.5%) compared to the control diet without the Ashwagandha fed a practical protein diet (35%). The formulation and proximate compositions for the experimental feed diets are presented in Table 1.

Table 1: Composition of experimental diets

Formulation				
Ingredients (%)	Diets (35% protein)			
	T ₀	T ₁	T ₂	T ₃
Ashwagandha root powder	0	2.5	5	7.5
GNOC	35	35	35	35
Fish meal	37	37	37	37
Wheat flour	10	9.5	9	8
Tapioca powder	10	9	8	7
Fish oil	4	4	3	3
Vitamin and minerals	4	3	3	2.5
Total	100	100	100	100

The experimental diets were prepared in the laboratory by using the Pearson square method. Roughly, raw materials were taken in the ground form and sieved and weighed on a digital weighing balance. The ingredients were mixed with the required amount of water (250 mL kg⁻¹). Then the mixture of feed was thermally processed in an autoclave at 121°C and 15 lbs; pressure for 10 – 15 minutes. After steam cooking of the feed mixture, the vitamin and mineral mixture was added and mixed well. Then the feed mixture with a hot plate extruded die and makes a pellet using a mechanical pelletizer. The feed pellets were sundried to reduce the moisture content to less than 10%. After that, the dried feed pellets were stored at room temperature in the differently labeled plastic containers of experiments with control diets in feed (2.5%, 5%, and 7.5%).

Shrimps were fed with experimental diets at 10% of their body weight till the end of the experiment. The daily-feed ratios were divided into four equal times at 8:00, 11:00, 14:00, and 17:00 hours to all aquaria. Aeration was provided more at night throughout the experiment for a better oxygenation level. After every sampling, the quantity of feed given was adjusted based on the increased weight.

Water quality management

By providing continuous aeration throughout the experimental period to maintain the optimum dissolved oxygen level in each aquarium. Before feeding, the

experimental tanks were cleaned manually and siphoning was done every day to remove excess feed and faecal matter from the formation of nitrogenous metabolites such as ammonia and nitrites. Around 10% filtered and disinfected seawater was replaced in the siphoned tank.

Growth performance and feed utilization

Every weekend and the end of the experiment, shrimps were collected from the experiment tank for sampling. Shrimp weight gain (WG), specific growth rate (SGR), Protein efficiency ratio (PER), Feed conversion ratio (FCR), and survival rate (SR) were calculated according to El-Sayed (1999) [11] from the sampling using the following formula: Mean weight increment = Final average body weight - Initial average body weight

$$\text{SGR}(\%/day) = \frac{\text{Final weight} - \text{Initial weight}}{\text{Experimental period}} * 100$$

$$\text{FCR} = \frac{\text{Feed intake (g)}}{\text{Weight gain (g)}}$$

$$\text{PER} = \frac{\text{Weight gain (g)}}{\text{Protein intake (g)}}$$

$$\text{Survival rate (\%)} = \frac{\text{No. of fish survived after rearing}}{\text{No. of fish stocked}} * 100$$

Analysis of Immuno-parameters of *P. vannamei*

At the final experimental stage, 100 µl haemolymph was collected from the ventral sinus of 10 shrimps of each experiment tank. The haemolymph collected in a 1 ml syringe (26 gauge) containing 900 µl anticoagulants solution (0.114 M Trisodium citrate [TSC] and 0.1 M Sodium chloride [NaCl], pH 7.45) for the analysis of Prophenol oxidase activity (PPO), Respiratory burst (NBT assay) and Total haemocyte count (THC) in the following procedures:

Prophenoloxidase (PPO) activity

The Phenoloxidase (PO) is the terminal enzyme in the ProPO system of the arthropod defense system which acts as both a recognition and effector component to promote cell-to-cell communication and subsequently eliminate the pathogens from the host. According to López *et al.*, 2003 [20], a sample of 50 µl was incubated for 3 minutes at 25°C with 50 µl of trypsin (0.1 mg/ml). After that 50 µl of L-DOPA (3 mg/ml in Cac buffer) was added and incubated for 10 minutes. The absorbance was measured at 490 nm (Hernández-López *et al.*, 1996) [13].

Respiratory burst (NBT assay)

According to the test of Song and Hsieh (1994) [37], 100 µl of HBSS was added as a blank solution to the first two wells in flat-bottomed microtitre plates. A collected haemolymph of 100 µl was added into the separate wells and incubated for 1 hour at 37°C for the adherence of cells to the microtitre plate. Then the remaining supernatant present in the plate was decanted and 100 µl NBT solution was added into each well and incubated for about 30 minutes at 37°C. After 30 minutes, the removal of NBT and 100 µl absolute methanol were added to the microtitre plates and waited for again 30 minutes. After the removal of absolute methanol, the cells

were washed gently 3 times with 100 µl of 70% methanol. At last, the entire methanol was removed, and also the addition of 120 µl KOH and 140 µl DMSO were added to the microtitre plate to dissolve the cytoplasmic formazan. Then, the optical density was measured at 630 nm in an ELISA reader.

Total haemocyte count (THC)

The total haemocytes present in the haemolymph were counted manually by using the haemocytometer under a microscope of randomly selected 25 squares.

Pathogenicity of *Vibrio parahaemolyticus* in *P. vannamei*

Preparation of *V. parahaemolyticus* stock culture

At the end of the 60-day experiment, 10 shrimps from each treatment were selected for the infection study. The bacterial strain of *V. parahaemolyticus* was collected from the College of Fisheries Science, GADVASU, Punjab, India. Isolated bacterial strains were cultured in Tryptic soy agar plates (TSA with 2.5% NaCl) for 24 hours at 25°C. Then the culture was transformed to 10 ml of Tryptic soy broth (TSA with 2% NaCl) and the broth was incubated at 25°C for 24 hours as a stock culture for the experiment.

Preparation of required density of *V. parahaemolyticus*

A stock culture *V. parahaemolyticus* was centrifuged in a refrigerated centrifuge at 3000 rpm for 10 minutes at 4°C. The supernatant is discarded and then resuspended in 50 µl PBS by adjusting the OD of the solution to 0.8 at 620 nm; corresponding to 6.4×10^8 cfu/ml. Serial dilution of *V. parahaemolyticus* using standard dilution technique with 50 µl to get the required bacterial concentration of 10^8 to 10^4 cfu/ml.

Injection of *Vibrio parahaemolyticus* in *L. vannamei*

A juvenile of *L. vannamei* in each treatment was subjected to challenge with *Vibrio parahaemolyticus* by injecting the bacterial suspension. The bacterial concentrations were injected at the second and third abdominal segments which contained 10^4 cfu/ml [LD₅₀ dose] PBS of 100 µl of bacterial suspension. On the negative side, the controlled juveniles were injected with only PBS and its susceptibility was conducted for 10 days.

Statistical analysis: The presented data are mean ± s.d. Data of mean growth, survival rate, immune responses, and disease resistance of shrimp in different formulated feed diets were tested for homogeneity and normal distribution by using one-way ANOVA. For the comparisons of different treatments, Duncan's multiple range test was used. The significance of each experiment was set as $P < 0.05$ (Snedecor and Cochran, 1994) [36].

Results

Physico-chemical water parameters

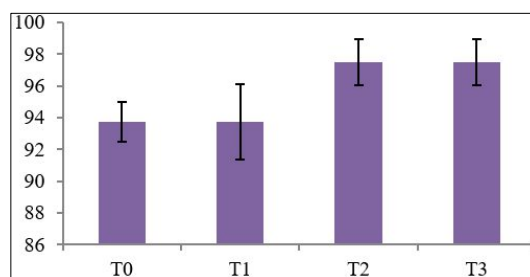
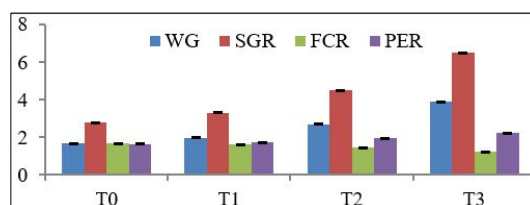
Physico-chemical water parameters including temperature, pH, dissolved oxygen, and alkalinity were recorded during the study period every week and shown in Table 2. The data of water quality parameters were kept in an ideal range throughout the experiment.

Table 2: Water quality parameters of different treatment

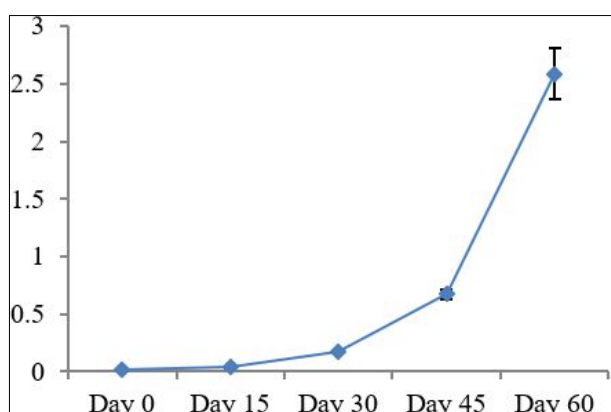
Water parameters	T ₀	T ₁	T ₂	T ₃
Temperature	29.71±0.3182	29.85±0.2449	30.04±0.2560	29.74±0.3021
pH	7.35±0.233	7.41±0.2748	7.31±0.1808	7.34±0.1996
Dissolved oxygen	5.91±0.221	5.92±0.2337	5.94±0.1701	5.91±0.2566
Alkalinity	124.6±1.5556	122.8±1.4856	123.5±1.1513	123.7±1.347

Growth performance

The results of growth performance are presented in Figure 2b and Table 3. The main effects of dietary supplementation with Ashwagandha (*Withania somnifera*) root powder in feed showed a significant increase in the growth level. The 20 shrimps from each treatment were selected for sampling. The initial mean weight of *P. vannamei* shrimp in all four treatments was ± 0.012 g. The final mean weight of *P. vannamei* in T₀, T₁, T₂, and T₃ were 1.686 ± 0.0053^a g, 2.004 ± 0.0135^b g, 2.722 ± 0.0051^c g, and 3.918 ± 0.0137^d g and the weight of *P. vannamei* in sampling was different in all treatments at every 15 days. As compared with other treatments, a significantly increased weight was observed in the T₃ treatment. The mean weight gain of *P. vannamei* at the end of the experiment (60 days), the significant weight gain difference ($p < 0.05$) among all the treatments, in which the increased weight gain (WG) in T₃ treatment in Figure 3. A significant difference ($p < 0.05$) of survival test observed in T₃ as compared to other treatments in Figure 2a and Table 3.

**Fig 2a:** Survivability rate of shrimps in different ARP feed diets**Fig 2b:** Growth performance (WG, SGR, FCR and PER) observed at different ARP treatments**Table 3:** Growth performance was observed in different ARP treatments

Treatments	T ₀	T ₁	T ₂	T ₃
Initial body weight (g/shrimp)	0.012±0.000	0.012±0.000	0.012±0.000	0.012±0.000
Final body weight (g/ shrimp)	1.686±0.0053	2.004±0.0135	2.722±0.0051	3.918±0.0137
WG	1.6742±0.005 ^a	1.9925±0.0135 ^b	2.7109±0.005 ^c	3.9065±0.013 ^d
SGR	2.790±0.008 ^a	3.320±0.022 ^b	4.518±0.008 ^c	6.510±0.022 ^d
SR	93.75±1.25 ^a	93.75±2.39 ^a	97.50±1.44 ^a	97.50±1.44 ^a
FCR	1.71±0.005 ^d	1.63±0.0023 ^c	1.47±0.0038 ^b	1.27±0.0022 ^a
PER	1.66±0.0052 ^a	1.74±0.0025 ^b	1.94±0.0050 ^c	2.23±0.0039 ^d

**Fig 3:** Mean weight gain (g) *P. vannamei* recorded in different treatments during culture period

Specific growth rate was calculated by using El-Sayed (1999) [11] as the data gained at the end of the 60-day experiment significantly ($P < 0.05$) higher SGR in treatment T₃ and lower SGR in treatment T₀ as shown in Figure 2b and Table 3.

Feed utilization

The FCR of *P. vannamei* was calculated at the feeding rate of 10% of body weight. The lowest feed conversion ratio was recorded in treatment T₃ in Figure 2b and Table 3. By

the statistical analysis, the FCR showed a significant difference ($P < 0.05$) among the treatments. In this study, FCR is inversely proportional with Ashwagandha root powder supplementation which directed the shrimp to a higher growth rate.

Challenge study

Disease challenge test of 0.1ml (1×10^4 cfu/ml) *Vibrio parahaemolyticus* in *P. vannamei* shrimps (20 numbers). The mortality rate in treatment T₃ showed lower mortalities after 10 days after the end of the experiment in Figure 4c and Table 5. The statistical analysis showed a significant difference ($P < 0.05$) between T₀, T₁, and T₃, but there is no significant between T₁ and T₂.

Immune response against *V. parahaemolyticus*

The result of immune-modulatory effects such as Prophenoloxidase activity (PPO), Superoxide anion production/ Respiratory burst (NBT assay) and total haemolytic count (THC) of dietary supplementation ARP in four treatments against *V. parahaemolyticus* were shown in the Figure 4a, 4b and Table 5. In the PPO test, the incorporated ARP in treatment T₃ showed the highest phenoloxidase significance ($p < 0.05$) than the control-fed diets. In Respiratory burst activity, the statistically significant difference ($p < 0.05$) in superoxide anion

production was found in various dietary ARP supplementation treatments. The treatment T₃ showed higher respiratory burst activity as compared to other dietary treatments. The highest total haemocytes count (THC) of

$8.88^d \times 10^6$ was recorded in T₃ followed by $7.94^c \times 10^6$ in T₂; $7.82^b \times 10^6$ in T₁ and $6.35^a \times 10^6$ in T₀ were shown in Table 3. A significant difference ($p < 0.05$) in total haemocytes was found in various dietary treatments of ARP.

Table 5: Immunological parameters and bacterial challenge test observed in different ARP treatments

Treatments	T ₀	T ₁	T ₂	T ₃
Immunological parameters				
PPO	0.154 ± 0.0006^a	0.162 ± 0.0004^b	0.171 ± 0.0008^c	0.185 ± 0.0023^d
Respiratory burst (NBT assay)	0.205 ± 0.0033^a	0.219 ± 0.0019^b	0.236 ± 0.0019^c	0.243 ± 0.0014^d
THC	6.350 ± 0.0294^a	7.820 ± 0.0443^b	7.947 ± 0.0085^c	8.885 ± 0.0340^d
Bacterial challenge test				
Mortality rate (MR)	72.5 ± 6.29^c	47.5 ± 4.78^b	35.0 ± 6.45^b	17.5 ± 4.78^a

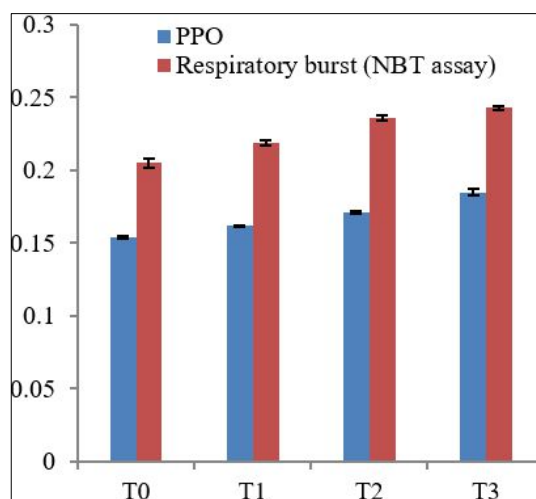


Fig 4a: Graphical representation of PPO and Respiratory burst (NBT assay) in different ARP feed treatment diet

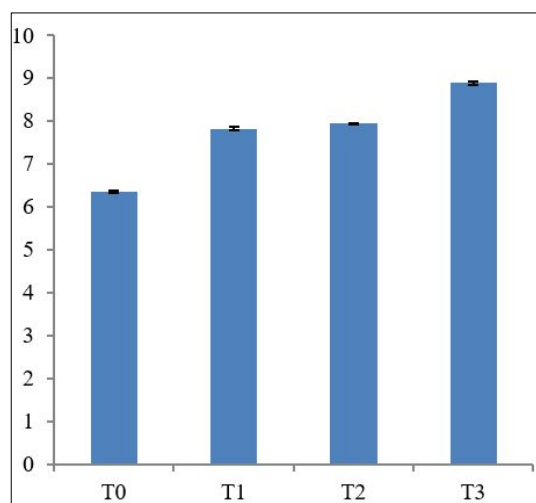


Fig 4b: Graphical representation of Total Haemocyte count in different ARP feed treatment diet

Discussion

Shrimps (*Penaeus vannamei*) and prawns (*Penaeus monodon*) in aquaculture have made significant advances in the last four decades in several parts of the world including India (Sahadevan *et al.*, 2019) [31]. The midpoint of host-environment-pathogens is the birthplace of disease occurrence. Disease arises due to the favourable environmental factors for the pathogenic microbes in aquatic systems, as well as the compromised immune defences of the host species. Among many pathogenic microbes, *Vibrio* species is peculiar for the

marine/brackishwater shrimps. This pathogenic microbe species majorly invades the chitin layer of the shell with its enzyme chitinase. At least 14 *Vibrio* species are implicated in disease outbreaks in shrimp, including *Vibrio harveyi*, *V. vulnificus*, *V. parahaemolyticus* & *V. cholerae* (Lara *et al.*, 2011) [18]. Ashwagandha (*Withania somnifera*) is a valued herb among the other herbs and it rejuvenates the cells and supports the immune systems to promote the species health. Ashwagandha mainly contains alkaloids and steroidal lactones especially withanolides (Withaferin A). Withaferin A inhibits notch-1 signalling and down-regulates pro survival pathways (Koduru *et al.*, 2010) [15]. Rao *et al.* (2020) [30] worked on the effect of dietary supplementation of Ashwagandha root extract in *L. vannamei* (0.05%, 0.1% and 0.15%) and documented that higher significance in growth performance and elevation of digestive enzyme activity ($p < 0.05$) at 0.05% among other treatment. Rana and Kumar (2020) [29] experimented with dietary feed supplementation of Ashwagandha root extract 0%, 1% and 3% in *Labeo rohita* and noticed that 3% diet supplementation having higher significance ($p < 0.05$) resulted in better FCR, growth rate, condition factor and higher protein retention. Bhavan *et al.* (2012) [4] observed higher survival and FCR rate in the experimental groups over control indicating the fact that the quality of PL was improved through herbal incorporated feeds. Srivastava *et al.* (2020) [39] studied the ARP supplementation (0%, 1%, 2% and 3% level) of *L. rohita* fingerlings. In which, the 2% treatment level demarcated the higher survival rate, growth rate and good FCR and PER; it showed that the best supplementation source for the grow-out stage of *Labeo rohita* fishes for improving growth performance and flesh quality. Citarasu *et al.* (2002) [8] found the same result that the Ashwagandha root powder incorporated diet increased the growth rate in *P. monodon*. Parmar (2020) [26] researched Nile tilapia fry (*Oreochromis niloticus*) on the different herbal powder concentrations in which the Ashwagandha fed diet treatments (0g/kg, 5 g/kg, 10 g/kg, 20 g/kg, 50 g/kg) respectively. The highest growth performance was observed in AT₃ as compared to other treatment levels against *Aeromonas hydrophila*. Akbary *et al.* (2017) [2] reported that SGR is increased in *Litopenaeus vannamei* fed with dietary mixture of four medicinal plants as compare to control diet. Laltlanmawia *et al.* (2019) [17] studied on ameliorating effects of a dietary mixture of *W. somnifera* root extract and vitamin C of different diet concentration treatment levels 0%, 0.01%, 0.1% and 1% in *L. rohita* against low pH and waterborne iron stresses. The treatment 1% diet significantly lowered the pH and decreased glucose, ALP, SGPT and SGOT levels compared to 0% treatment level. Zahran *et al.*

(2018) ^[43] studied the dietary effects of *Withania somnifera* 2.5% and 5% to evaluate the immune intensification and antioxidant response against *Aeromonas hydrophila* in Nile tilapia; noticed that reduced mortality by 20% and increased immune factors at 5% diet supplementation.

Suphantharika *et al.* (2003) ^[40] exposed a significant improvement in PO action in all hemocytes treated with yeast-derived products as compared to control. Ahilan and Nithiyapriyatharshini (2015) ^[1] studied the influence of herbal additives (garlic, ashwagandha and tulsi) on the growth and disease resistance of goldfish (*Carassius auratus*). Ashwagandha at 1.0% concentration yielded the highest mean weight gain (2.346 g). The phenoloxidase activity of prawn-fed glucan and biogut-added diets was significantly higher than that of prawn-fed with a control diet (Pavadi, 2007) ^[27]. The phenoloxidase activity of prawn-fed chitin incorporated @ 0.75% was significantly more (Naveen Kumar, 2008) ^[25]. Borkar *et al.* (2014) ^[6] studied on anti-inflammatory activity of *W. somnifera* leaf extract in stainless steel implant-induced inflammation in adult zebrafish. The thin layer chromatography (TLC) separated part of *W. somnifera* supernatant has a significant inhibitory effect on TNF α in adult zebrafish.

Anne (2011) ^[3] worked on growth performance of *M. rosenbergii* post larvae (PL) fed with *Ocimum sanctum* (Tulsi) and *W. somnifera* (Ashwagandha) incorporated in feed. Among the two herbs, the PL fed with *W. somnifera* incorporated diet has exhibited better growth performance followed by *O. sanctum*. Takahashi *et al.* (2000) ^[41] reported that oral administration of LPS to viral challenged kuruma shrimp (*Penaeus japonicus*) highly influenced by the increasing activity of proPO system and improves the controlling capacity of virus. Harikrishnan *et al.* (2012) ^[12] studied on immunomodulatory effect of *W. somnifera* (0.1% and 1%) supplementation diet in the giant freshwater prawn, *Macrobrachium rosenbergii* against *A. hydrophila*. The total haemocyte count and innate immune response have higher significance ($p < 0.05$) than the controlled diet. Sharma *et al.* (2010) ^[33] worked on the effect of *W. somnifera* root as a feed additive in diet (0g/kg, 1g/kg, 2 g/kg, 3g/kg) on immunological parameters and disease resistance to *Aeromonas hydrophila* in *Labeo rohita* fingerlings.

Trivedi *et al.* (2024) ^[42] studied the dietary inclusion of medicinal herbs like Ashwagandha 'A' (*Withania somnifera*) and Shatavari 'S' (*Asparagus racemosus*) in *Channa punctatus* and resulted that higher significance ($p < 0.05$) of growth and digestive enzyme activity in AS3 (3% of A and S). Dietary *W. somnifera* at the level of 2 g/kg showed significantly ($p < 0.05$) higher immunological parameters protection against *A. hydrophila* infection than control. Kour (2007) ^[16] studied the effects of Ashwagandha (*W. somnifera*) and Satavari (*Asparagus racemosus*) on growth and colouration in certain freshwater ornamental fishes. The higher weight gain was found in koi carp and in silver molly with the effect of the mixture of Ashwagandha (0.08 g/kg body weight/day) and Satavari (0.2 g/kg body weight/day).

Conclusion

The results of the present study reveal that different dietary supplementations of Ashwagandha root powder could effectively influence the growth performance (WG, SR, SGR) be the highest rate and increase the immunological parameters such as prophenoloxidase activity (PPO),

respiratory burst (NBT assay) against *Vibrio parahaemolyticus* and increase the protein efficiency ratio as well as total haemocyte count and also reduced the feed conversion ratio. The current study documented an experiment on *Litopenaeus vannamei* fed with various dietary supplementations (0%, 2.5%, 5%, and 7.5%) of Ashwagandha root powder incorporated feed. Among these, 7.5% Ashwagandha root powder supplemented diet satisfied the above results. By accomplishing, this also increases the resistance against *V. parahaemolyticus* challenge as compared to shrimps fed with a control diet.

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