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Effect of dietary supplementation of Aswagandha (*Withania somnifera)* root extract on growth performance, digestive enzymes activities of white leg shrimp *Litopenaeus vannamei*

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Abstract
In the present study the Ashwagandha (*Withania somnifera*) root extract was incorporated in the formulated diet at the levels of 0.05%, 0.1% and 0.15% to understand its effect on growth and digestive enzyme activities in *Litopenaeus vananmei.* The experiment was carried out for a period of 91 days under laboratory conditions with triplicate. The control and experimental diets were prepared to have almost similar crude protein level of 35% using the ingredients such as fish meal, shrimp meal, soya meal, deoiled rice bran, ground nut oil cake as major ingredients. Uniform water salinity of 15 ppt was maintained in the control as well as the experimental tanks. A significant improvement (P<0.05) in growth performance (weight gain, specific growth rate, feed conversion ratio and protein efficiency ratio) and elevation in activities of digestive enzymes (protease, amylase and lipase) were recorded in *L. vannamei* fed with Ahwagandha (*Withania somnifera*) incorporated diet compared to those of control. The treatment containing diet with Ashwagandha @ 0.05% was found significantly superior among all the treatments. The results indicated that inclusion of Ashwagandha in *L. vannamei* diets can enhance the growth parameters and activities of digestive enzymes.

Keywords: Aswagandha, *Withania somnifera*, *Litopenaeus vannamei*

1. Introduction
India is the second largest country in aquaculture production in the world. Pacific white leg shrimp, *Litopenaeus vannamei* has become the primary species currently being cultured in India. The total shrimp, predominantly the pacific white leg shrimp (*Litopenaeus vannamei*), produced through brackish water aquaculture in India during the year 2017-18 was 6,22,327 tonnes, from 93,496 ha area, accounting to a productivity level of 6.6t/ha/yr [1]. Intensive farming with high stocking density is the most preferred technique in of *L. vannamei* shrimp farming practice. As microbial disease in shrimp culture implies serious financial loss, an indiscriminate use of antibiotics for disease treatment may lead to the promotion of undesirable side effects. Several alternative methods are considered to improve the quality and sustainability of aquaculture production [2]. Of these methods, a wide range of medicinal plants show potential effects on growth and survival as well as on the antimicrobial properties of aquatic organisms [3]. This leads to the belief that phytochemicals could be an alternative to the chemotherapeutic molecules in aquaculture [4]. Medicinal plants include herbs, spices, seaweeds, herbal extracted compounds, traditional Chinese medicines, and commercial plant-derived products

A number of novel feed additives has been derived from specific natural compounds mostly derived from yeast and herbal extracts, so called “phytobiotics”, capable of modulating the microflora towards a favorable composition, favoring the development of beneficial bacteria and inhibiting potentially pathogenic micro-organisms and parasites. The ban on not to use certain antibiotic growth promoters in aquaculture and the subsequent search for alternatives, has revealed the potential of phytobiotic products on gut health, feed efficiency, overall performance and productivity [5].

In the view of the above, the Indian medicinal plant Ashwagandha (*Withania somnifera*) belongs to the family Solanaceae has been selected and incorporated with formulated feeds.

The root of Ashwagandha (*W. somnifera*) the 'Indian ginseng' is also known as winter cherry has been an important herb in the ayurvedic, siddha and unani medical systems for over 3000 years in the treatment of various physiological disorders. Withanolides are the major active constituent of Ashwagandh (*W. somnifera)*. The roots of the plant are categorised as rasayanas, which are reputed to promote health and longevity by augmenting defence against disease, arresting the ageing process, revitalising the body in debilitated conditions, increasing the capability of the individual to resist adverse environmental factors and by creating a sense of mental wellbeing.

In order to promote the growth and survival of *L. vannamei* in a sustainable manner, the present study was conducted with an objective to evaluate the effect of Ashwagandha on growth performance and digestive enzymes activities of *L. vannamei* through dietary supplementation.

2. Materials and Methods

2.1. Experimental set up and shrimp

The experiment was conducted in Wet Laboratory of the Department of Aquaculture, College of Fishery Science, Sri Venkateswara Veterinary University, Muthukur, Nellore District for a period of 127 days (including acclimatization period of 36 days). *Litopenaeus vannamei* post laravae were obtained from CP Shrimp Hatchery, Kothakoduru, Thotapalliguduru Mandal, Nellore District, which has been authorized by Coastal Aquaculture Authority (CAA), Chennai to produce seed. Post larvae transported by road in plastic bags containing 30 ppt saline water were transferred to the same salinity water in to the wet lab. The animals were acclimatized to experimental salinity (15 ppt) by stepwise decrease at the rate of 2‰ per day from the original salinity, by the addition of freshwater. Upon completion of acclimatization, all the shrimps were reared in the same experimental condition for about 36 days. During this period larvae were fed with control diet. The experiment was carried out during the period from November, 2019 to March, 2020. The glass tanks of size 60x30x30 cm used for the experiments. The tanks were filled with 15 ppt saline water and provided with aeration. The water in all the tanks was checked daily for various water quality parameters. Ten numbers of Shrimps with initial average weights of 2.03±0.06 gm were introduced in to each aquarium tank and triplicates were maintained for each treatment including control. Regular water exchange of 25% was done every day. Left over feed, excreta and other debris were siphoned off from the bottom of the tank without disturbing the shrimps.

2.2. Experimental Feed preparation and Feeding

In the experiment, formulated feeds with the crude protein 35% were used for feeding. The experimental diets were prepared by incorporating Ashwagandha extract @ 0.05%, 0.10%, 0.15%. The control diet was without Ashwagandha extract. Ashwagandha root extract was procured from M/s. Naturalle herbal feed company, Nellore. The major ingredients used in the feed and all the experimental feeds were estimated for proximate composition (AOAC, 1995). Required quantity of each ingredient was weighed and ground into powder and sieved. All the ingredients were then mixed in required proportion and water was added at the rate of 30 ml per every 100g of feed and dough was prepared. Maida (1%) was used as a binding agent in the feed. The dough was cooked for 20 minutes in pressure cooker and then cooled. Vitamin premix, mineral premix, soya lecithin, fish oil were added. Ashwagandha root extract was added as per the requirement of respective experimental feed. The homogenous dough was pressed through a pelletizer (La Monferrina S.R.L., Italy) with a sieve of 1 mm diameter. The feed was dried in shade and then in hot air oven at 80-90^{0}C to reduce the moisture content to 10% and stored properly in dry and air tight bottles and kept in dark cool place. Shrimps were fed at the rate of 6% body weight initial days and then reduced to 2% based on the average body weight. The pelleted feed was made into small granules of 1mm size and fed four times a day (7.00AM, 11.00AM, 3.00PM and 7.00PM). Left over feed and feces in all the aquarium tanks were removed after two hours of feeding.

Table 1: Composition of Experimental Diets (as % Feed basis)

Ingredient	Control	0.05% Ashwagandha Extract	0.1% Ashwagandha Extract	0.15% Ashwagandha extract
Fish meal	20	20	20	20
Soya meal	20	20	20	20
Shrimp meal	10	10	10	10
GNOC	10	10	10	10
DOB	26	26	26	26
Maize	8	7.95	7.9	7.85
Maida	2	2	2	2
[a]Vitamin premix	1	1	1	1
[b]Mineral premix	1	1	1	1
Fish oil	1	1	1	1
Soya lecithin	1	1	1	1
Ashwagandha root extract	0	0.05	0.1	0.15

[a] Vitamins (mg kg-1): Vitamin A 20.0, Vitamin D 4.0, Vitamin E 120.0, Vitamin K 60.0, Choline chloride 6000.0, Thiamine 180.0, Riboflavin 240.0, Pyridoxine 180.0, Niacin 1080.0, Pantothenic acid 720.0, Biotin 2.0, Folic acid 30.0, Vitamin B12 0.150 Inositol 1500.0, Vitamin C 9000.0.

[b] Minerals (g kg-1): CaCO3 28.0, K2SO4 10.0, MgSO4 12.5, CuSO4 0.2, FeCl3 0.5, MnSO4 0.5, KI 0.01; ZnSO4 1.0, CoSO4 0.01, Cr2SO4 0.05, Bread flour 7.14

Table 2: Proximate composition of Experimental Diets (as % dry basis)

Ingredient	Control	0.05% Ashwagandha extract	0.1% Ashwagandha Extract	0.15% Ashwagandha extract
Moisture	9.46±0.085	9.35±0.083	9.43±0.047	9.42±0.001
Crude protein	35.52±0.406	35.10±0.226	35.41±0.586	35.07±0.297
Ether extract	5.57±0.096	5.80±0.135	5.84±0.092	5.68±0.105
Crude fiber	5.36±0.078	5.28±0.085	5.44±0.107	5.37±0.097
Total ash	13.46±0.115	13.66±0.105	13.56±0.136	13.61±0.099
NFE	40.09	40.16	39.75	40.27

*Nitrogen free extract NFE was calculated by difference = 100 - (moisture% + Crude protein% + Crude fibre% + Ether extract% + Total ash%).

2.3. Growth performance

The shrimps from each aquarium tank were weghed for estimating growth performance by taking their total body weight.

Weight gain (gm) = Final body weight (gm) – Initial body weight (gm).

Specific growth rate (SGR) = (log of final weight – log of initial weight) / no. of days x100

Feed Conversion Ratio (FCR) = Feed given (dry weight)/ Body weight gain (wet weight)

Protein Effeciency Ratio (PER) = Weight gain (gm)/ Crude protein fed (gm)

2.4. Physico-Chemical parameters of water

The important water quality parameters like water temperature, pH, dissolved oxygen, ammonia and nitrite were recorded weekly during the experimental period following the standard methods. All water quality parameters were found to be with in the normal range for rearing *L. vannamei.*

2.5. Digestive enzyme assay

For estimation of digestive enzyme activity of shrimp, the crude extract of whole gut and hepatopancreas was used. The whole Gut and hepatopancreas was dissected out and were homogenized with cold deionized water (1:10). Later, the homogenized mixture was centrifuged at 5000 g for 20 min at 4 °C. The clear supernatant was separated carefully and was passed through a membrane filter (0.45mm pore size). Aliquots of the samples were made in 1.5 ml Eppendorf tubes in triplicates and stored at -20 °C for analyzing different digestive enzyme activity.

2.5.1 Specific protease activity was assayed using casein digestion method following the recommendations of Drapeau [6]. For the reaction 0.1 ml tissue homogenate was incubated at 37 °C for 15 min with 2.5 ml of 1% (w/v) casein and 0.05 M Tris-HCl (pH 7.8). In order to stop the reaction, 2.5 ml of trichloroacetic acid (TCA) was added to the mixture and the reaction mixture was subsequently filtered. For blank un incubated reaction mixture was used. The absorbance was recorded at 280 nm against the blank. Protease activity was expressed as micromole of tyrosine released mg^{-1}protein min^{-1} at 37 °C.

2.5.2 Specific amylase activity was estimated by using 3,5-dinitrosalicylic acid (DNS) method [7]. For reaction 0.1 mL of crude extract (sample), 0.1 mL of 1% (w/v) of starch solution and 1.8 mL phosphate buffer (pH 7.5, 0.1 M) was used. The above mixture was incubated for 30 min at 37 °C. 2 mL of a 3,5-dinitro salicylic acid (DNS) solution was added to the reaction mixture and maintained in a water bath for 5 minutes in order to stop the reaction cycle. Absorbance of the reaction mixture was recorded at 570nm. Total amylase activity was determined from the maltose standard curve and the value was expressed as mg of maltose released at 37 °C per min per mg of protein.

2.5.3 Specific lipase Activity was determined based on the suggestion of Cherry and Crandall [8]. The reaction mixture consists of distilled water, tissue homogenate, phosphate buffer (0.1M, pH 7) and olive oil emulsion as substrate. Above reaction mixture was incubated at 27 °C for 24 h. Following which, 95% alcohol and two drops of phenolphthalein indicator were added and titrated against 0.05 N NaOH until the appearance of permanent pink colour. For blank, the mixture was inactivated by keeping for 15min in boiling water bath prior to addition of buffer and olive oil emulsion. The milliequivalent of alkali consumed was taken as lipase activity.

3.

2.6 Statistical Analysis

Values for each parameter measured were expressed as the arithmetic mean ± standard deviation (SD). Statistical analysis of data involved analysis of variance (ANOVA) followed by the comparision of means following Randomised Block Design (RBD) at 5% level of significance. The software programme of Web Agristat 2.0 package was used for the analysis.

3. Results and Discussion

Feeding of Ashwagandha extract supplemented diet to *L. vannamei* led to significantly (p<0.05) increased growth rate (weight gain and SGR) compared to control. FCR and PER were significantly (p<0.05) better than control in all the treatments receiving diet supplemented with Ashwagandha exract (Table 3). Overall the survival percentage varied from 73.33 % in control and 93.33% to 100% in treatment groups. After final sampling, highest survival recorded was 100% in diet containing Ashwagandha 0.05% fed group and lowest was 73.33% in control.

Table 3: Growth parameters of *L. vannamei* in various treatments of dietary supplementation of Ashwagandha

Parameter	Control	0.05% Ashwagandha Extract	0.1% Ashwagandha Extract	0.15% Ashwagandha extract
Initial	2.10±0.148	1.96±0.056	2.01±0.132	2.05±0.036
Final weight	15.74±1.006	18.48±0.926	17.94±1.493	17.72±1.299
Weight Gain	13.64±0.895	*16.52±0.901	15.93±1.451	15.67±1.308
SGR	2.22±0.049	*2.46±0.046	2.40±0.093	2.37±0.089
FCR	1.95±0.122	*1.61 ±0.085	1.67±0.156	1.7±0.147
PER	1.45±0.096	*1.77±0.1	1.70±0.160	1.68±0.142
Survival	73.33	100	93.33	93.33

The digestive enzymes such as protease, amylase and lipase were significantly (p<0.05) higher in Ashwagandha extract incorporated feed fed shrimps when compared to that of control feed fed shrimps (Table 4). The protease, amylase and lipase activities were highest in L. vannamei fed with diet containing 0.05% of Ashwagandah root extract compared to control and other treatments.

Table 4: Digestive enzymes activities of *L. vannamei* in various treatments of dietary supplementation of Ashwagandha.

Treatments	Protease activity (U/mg protein)	Amylase activity (U/mg protein)	Lipase activity (U/mg protein)
Control	1.126±0.13	0.222±0.019	0.00413±0.0273
O.05% Ashwagandha root extract	1.797±0.04*	0.250±0.016*	0.00520±0.000383*
0.1% Ashwagandha root extract	1.683±0.04	0.247±0.019	0.00511±0.00024
0.15% Ashwagandha root extract	1.657±0.015	0.242±0.015	0.005073±0.000206

The herbal bio-medicinal active principles in the aquaculture of shrimps that have the characteristics of growth promoting ability, tonic to improve the immune system, antimicrobial capability, stimulating appetite and antistress characteristic due to the active principle natures such as alkaloids, flavonoids, pigments, phenolics, terpenoids, starch, steroids and essential oils will be of immense use in the culture of shrimps. This practice will reduce the side effects of applying the synthetic compounds and the cost and also make it eco-friendly. Hence, the alternative herbal biomedicines prove to be very effective in aquacultural operations [9]. The results of present experiment indicated significant (<0.05) improvement of growth and digestive enzymes activities in *L. vannamei* fed with Ashwagandha (*W. somnifera*) root extract incorporated feed. Therefore, it is evident that incorporation of herbal extracts such as Ashwagandha root extract inclusion in the feed stimulates productivity of the shrimp farm.

In the present study there was significant (<0.05) increase in body weight gain, SGR, PER and better FCR in *L. vannamei* fed with Ashwagandha root extract compared to control. Similarly there was also significant (<0.05) increase in digestive enzyme activities (Protease, Amylase and Lipase) compared to control. The enhanced growth performance in present study may be attributed to increased secretion of digestive enzymes, which facilitated the break down of food materials and availability of nutrients for absorption. Similar results were obtained with Ashwagandha fed diet in *M. rosenbergii* [10]. Improved growth, SGR, PER and digestive enzyme activity in *L. vannamei* fed with equal amount of dietary mixture of four medicinal plants was reported by Akbary *et al.* [11] Muralisankar *et al.* [12] clearly demonstrated that the dietary supplementation of dried leaf powder of herbs, such as *O. sanctum*, *S. trilobatum*, and *P. amarus* influence food intake, survival rate, growth rate, energy utilization and vitamin levels and activities of digestive enzymes of freshwater prawn. There was a significant (P<0.05) improvement of digestive enzyme activity (amylase, protease and lipase), feed intake and conversion and production efficiencies in P. monodon post larvae fed with different percentages (0, 25, 50, 74 and 100%) of the herbal appetizer *Z. officinalis* enriched Artemia [13]. Significant elevations (P<0.05) in weight gain, activities of digestive enzymes (Protease, Amylase, Lipase), concentration of total protein were recorded in *Mystristica fraganns* incorporated feed fed *M. rosenbergii* post larvae followed by *Glycyrrhiza glabra* and *Querccus infectoria* when compared with control [14]. Increase in avtivities of protease, amylase and lipase was also reported in *M. rosenbergii* PL fed with *A. paniculata, C. quadragularis* and *E. alba* incorporated feed [15] and *M. koenigii, C. sataivum* and *M. arvensis* incorporated feed [16]. Yu *et al.* [17] reported that the combined Medicinal herb and *Bacillus* in diet could enhance growth in *L. vannamei* because it improve digestive enzyme activity and digestive metabolism.

Several researchers also reported that with the increasing body weight gain there is significant (<0.05) increase in feed intake in different Ashwagandha fed groups [18, 19]. Significant (<0.05) improvement in body weight was found in *Channa punctatus* fed with Ashwagandha and Shatavari incorporated diets [20]. Kim *et al.* [21] suggested that unknown factors in various medicinal herbs led to favorable results in fish trials. Flounder (*Paralichthys olivaceus*) fed a diet containing Chinese herbs had increased pepsin activity in the stomach and protease activity in the intestines [22].

Presence of certain withanolides and saponins in the plant extracts is reported to promote growth by enhancing the feed conversion efficiency, this may be the possible reason for higher growth observed in the present study as Ashwagandha root extracts used in this study were contained 5% withanolides. The present study clearly highlighted the potential value of Ashwagandha root extract @ 500mg/kg diet (0.05%) that enhanced the growth performance and diegestive enzyme activity of *L. vannamei*.

4. Conclusion

As many factors could influence the effectiveness of Medicinal herbs, more practical evaluation should be done to discover these. In the current study, the incorporation of Ashwagandha root extract in the diet promoted the growth performance of *L. vannamei* by improving digestive enzyme activity. This finding indicates that Ashwagandha root extract is positive dietary additives to induce effective technical and economical propagation of cultured shrimp.

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