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## Dietary ashwagandha, *Withania somnifera* (L. dunal) potentiates growth, haemato-biochemical response and disease resistance of *Labeo rohita* (Hamilton, 1822) against *Aeromonas hydrophila* infection

**Arun Sharma, Thomgam Ibemcha Chanu and Ashutosh D Deo**

### Abstract

A 42 days experiment was conducted to understand the role of *Withania somnifera* (ashwagandha) administered through feed in growth, haemato-biochemical response and disease resistance of *Labeo rohita* (Hamilton, 1822) against *Aeromonas hydrophila* infection during the period from February to March, 2009 at Department of Aquatic Health and Environment (AHE), College of Fisheries Lembucherra, Tripura, India. *W. somnifera* fed at 0, 0.1, 0.2 and 0.3 g/100 g of feed for 42 days to *L. rohita*, led to enhance disease resistance against experimental infection with *A. hydrophila*. At 0.2 g ashwagandha/100 g of feed, there was an increase in survivability ( $43.00 \pm 0.75\%$ ) compared with the control ( $2.00 \pm 0.03\%$ ). Moreover, there was a significant increase in growth and feed conversion. There were a significantly higher number of erythrocytes, leucocytes, haemoglobin, haematocrit value, neutrophils, lymphocytes, globulin, A/G ratio and enhanced bactericidal activities compared with the control following feeding with *W. somnifera*. This suggests that *W. somnifera* at 0.2 g/100 g of feed can be used as dietary supplement to improve the growth, haemato-biochemical response and disease resistance against *A. hydrophila* for *L. rohita* fingerlings.

**Keywords:** *Aeromonas hydrophila*, *Withania somnifera*, *Labeo rohita*, immunity, disease resistance

### 1. Introduction

Farmed fish are prone to inevitable stresses and thus increased susceptibility to disease particularly bacterial haemorrhagic septicaemia caused by *Aeromonas hydrophila* occurs worldwide in freshwater fish [1]. To date, disease control strategies for these pathogens have been centred on the use of antibiotics and chemotherapeutics which has been widely criticized for their negative impacts [2]. Furthermore, pathogen like the bacterium *A. hydrophila* no effective vaccines available due to the complex antigenic structure [3]. Therefore, attention has focused on immunostimulant of plant origin which have been reported to possess various activities like antistress, growth promoters, antimicrobial and immunomodulatory [4].

In particular, the Indian medicinal plant *Withania somnifera* a time honoured Ayurvedic herbal immune booster and known as Indian ginseng [5] to be effective as an immune-modulatory agent in animals, including fish [6, 7]. Number of active principles have so far been isolated from *Withania somnifera* and have been reported to possess several medicinal properties like anti-stress [8], anti-oxidant [9], rejuvenating [10] and immunomodulatory [11]. Withanolides are the major active constituent of *Withania somnifera* that are isolated from its root and leaves however, the roots are the major portions of the plant used therapeutically [12].

As immunological studies continue to include dietary manipulation, biochemical profiles in fish and other aquatic organisms serve as important bio-indicators for monitoring of aquatic environment [13, 14, 15] and haematology becomes a necessary research tool for further interpretation of dietary effects, an index of the physiological status of fish [16, 17, 18]. In addition, the biochemical and haematological indices is direct indicator of high risk and lessen the immune system, ultimately increasing disease prevalence [19].

Akotkar *et al.* [20] revealed significant effect on body weight gain, feed consumption and feed conversion efficiency in different dietary treatments of *Withania somnifera* in broilers. Similarly, Khobragade [21] reported significant rise in body weight, body weight gain and

haemato-biochemical parameters on supplementation of medicinal plants *Tinospora cordifolia* and *Leptadenia reticulata* in broilers. The supplementation of *Gynostemma pentaphyllum*, a traditional Chinese herbal medicine, to grass carp feed resulted in increased weight gain, feed conversion efficiency and specific growth rate [22]. Against this background, an effort has been made to evaluate the potential of dietary *W. somnifera* in growth, haemato-biochemical response and disease resistance against *A. hydrophila* infection in *Labeo rohita* (Hamilton) fingerlings.

## 2. Materials and methods

**2.1 Fish and experimental setup:** The experiment was carried out during the period from February to March, 2009 at Department of Aquatic Health and Environment (AHE), College of Fisheries Lembucherra, Tripura, India. *Labeo rohita* fingerlings with an average weight of  $18.43 \pm 0.5$  g were obtained from the College of Fisheries fish farm, Tripura, India. The fish were transported in a 500 L container with sufficient aeration to the wet laboratory of the college. Next day, the fish were given a mild salt treatment (1%) for 5 min to ameliorate the handling stress and then acclimatized to the laboratory conditions for 15 days. Two hundred and forty fingerlings of *L. rohita* were randomly distributed in four treatment groups each with three replicates following a completely randomized design (CRD). The fingerlings were fed to satiation and the feeding trial was conducted for 42 days.

**2.2 Bacterial culture:** Pathogenic strain of *Aeromonas hydrophila* was received from the Aquatic Environment and Health Management Division, CIFE, Mumbai. *A. hydrophila* was grown on nutrient broth (HiMedia Ltd., India) for 24 hours at 37 °C. The culture broth was centrifuged at 3000 x g for 10 min. The supernatant was discarded and the pellets were resuspended in sterile phosphate buffer saline (PBS, pH 7.4) and the OD of the solution was adjusted to 0.5 at 456 nm, which corresponded to  $1 \times 10^7$  cells/mL.

**2.3 Feeding regimes:** The plant of *Withania somnifera* was collected from the local farmers of west Tripura district and was scientifically identified. The roots were collected from the plants and washed thoroughly with tap water to get rid of dirt. After washing, the roots were dried under shade to make them suitable for grinding. The dried plant roots were grounded in a mechanical grinder and sieved. The powder obtained was stored in an air tight container for further use. The experimental diet was prepared with the locally available ingredients containing 0 g (control), 0.1 g, 0.2 g and 0.3 g *W. somnifera* root powder/100 g of feed (Table 1). Initially all ingredients were mixed thoroughly by adding water, and then made into pellets by using a hand pelletizer [23] and then dried at 40 °C for 12 hours. Challenge was done by intraperitoneal injection with 0.2 mL of bacterial suspension. Mortality was observed for all groups for 14 days. The confirmation of the infection was accomplished after re-isolating the bacteria from the dead fishes. The relative percentage survival (RPS) was calculated according to Misra *et al.* [24]. Sub-groups of 10 fish were used to determine growth performance in which the percentage weight gain, specific growth rate (SGR) and feed conversion ratio (FCR) were determined for each group according to Choudhury *et al.* [25].

Weight gain% =  $\frac{\text{Final Weight} - \text{Initial Weight}}{\text{Initial Weight}} \times 100$

SGR =  $\frac{\log \text{ of final weight} - \log \text{ of Initial Weight}}{\text{no. of days}}$

FCR =  $\frac{\text{feed given (dry weight)}}{\text{body weight gain (wet weight)}}$

**Table 1:** Composition of experimental diets (g/100 g of feed).

Ingredients	Control	0.1	0.2	0.3
Wheat flour (g)	60	58.9	57.8	56.7
Fish meal (g)	35	35	35	35
Vitamin-mineral mix (g)	3	3	3	3
Cod liver oil (ml)	2	2	2	2
Ashwagandha root powder (g)	0	0.1	0.2	0.3

**2.4 Physico-chemical water parameters:** Selected water quality parameters like water temperature, alkalinity, dissolve oxygen, pH, ammonia- nitrogen (NH<sub>3</sub>-N), free carbon dioxide and total hardness were recorded at 14 day intervals during the experimental period following the standard methods [26]. All water quality parameters were found to be within the normal range for rearing of *Labeo rohita*. At the end of the feeding trial, five fish from each replicate for a total of fifteen fish from each treatment were anaesthetized using CIFECLAM (50µl/L). The blood was collected by venepuncture, using a 24 gauge needle, previously rinsed with 2.7% EDTA solution. Blood collected was transferred immediately to a test tube containing a small amount of EDTA powder (as an anticoagulant) and shaken gently to prevent haemolysis of blood cells. The blood samples were used for determination of haemoglobin content, haematocrit value, total leucocyte count, total erythrocyte count and blood glucose. For serum assay, five fishes from each replicate were anaesthetized, the blood was collected without anticoagulant, allowed to clot for 2h and then centrifuged at 3000 x g for 10 minutes followed by collection of serum with a micropipette. Serum was stored at -80 °C until use.

**2.5 Haematological parameters:** Blood hemoglobin was estimation by Cyanmethemoglobin method [27] using Drabkins fluid (Qualigens diagnostics, India). The absorbance was measured using a spectrophotometer at 540 nm and the final concentration was calculated by comparing with a Cyanmethemoglobin standard (Qualigens diagnostics). The haemoglobin concentration was then calculated by using the following formula: Haemoglobin (g /dL) =  $60 \left[ \frac{\text{OD (T)}}{\text{OD (S)}} \right] \times 251/1000$ . Where OD (T) is absorbance of test and OD (S) is absorbance of standard. The number of total erythrocyte and leucocyte were counted according to Gupta *et al.* [27]. Red blood cell diluting fluid (Qualigens Fine Chemicals, India) and WBC diluting fluid (Qualigens Fine Chemicals, India) were used for total erythrocyte counts and total leucocyte counts, respectively. Twenty microlitres of blood was used with 3980 µL of corresponding diluting fluid in a clean test tube and shaken well to suspend the cells uniformly in the solution. Cell counts were made using a Neubauer's counting chamber. Cell numbers were calculated according to the following formula: No. of cells (cu./mm) =  $\frac{\text{no. of cells counted} \times \text{dilution}}{\text{area counted} \times \text{depth of fluid}}$ . The Haematocrit value was determined for each group according to Anderson and Siwicki [28] with partial modification. Blood was drawn into the graduation mark 100 on the heparinized hematocrit pipette. Both the openings of the pipette were closed with rubber stoppers and centrifuged for 3 minutes. After centrifuging, the capillary tubes were placed on a reading device and the volume was recorded. The haematocrit value was expressed as the percentage fraction of blood cells in the total volume (volume %).

**2.6 Biochemical parameters:** Serum protein was estimated by Biuret method [29] using a total protein kit (Crest Biosystems, India). Albumin was estimated by the

bromocresol green (BCG) dye binding method [30]. The absorbance of standards and tests were measured against the blank in a spectrophotometer at 630 nm. Globulin was calculated by subtracting albumin values from total serum protein. The albumin/globulin (A/G) ratio was calculated by dividing albumin values by globulin values. Blood glucose was estimated by GOD/POD method [31] using a Glucose diagnostic kit (Crest Biosystems, India). Serum alanine aminotransferase (ALT) activity and aspartate aminotransferase (AST) activity was estimated [32] using serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) diagnostic kit (Crest Biosystems, India). Serum Alkaline Phosphatase activity was estimated [33] using Alkaline Phosphatase diagnostic kit (Crest Biosystems, India).

**2.7 Serum bactericidal activity:** The serum bactericidal activity was measured by following the method of Rao *et al.* [34] with partial modification. *Aeromonas hydrophila* bacterial culture was centrifuged and the pellet was washed and suspended in PBS. OD of the suspension was adjusted to 0.5 at 546 nm. This bacterial suspension was serially diluted (1:10) with PBS five times. Serum bactericidal activity was determined by incubating 2 µl of this diluted *A. hydrophila* suspension with 20 µL of serum in a micro-vial for 1 h at 37 °C. In the bacterial control group, PBS replaced the serum. After incubation, the number of viable bacteria was determined by counting the colonies grown on nutrient agar plate for 24 h at 37 °C.

**2.8 Statistical analysis:** Values for each parameter measured were expressed as the arithmetic mean ± standard error (SE). Statistical analysis of data involved one way analysis of

variance (ANOVA) followed by the comparison of means following Least Square Design (LSD) at 5% level of significance. The software program SPSS (Version 15.0; SPSS) for Windows was used for the analysis.

### 3. Results

**3.1 Growth performance:** Feeding of ashwagandha supplemented diet to *L. rohita* led to significantly increase growth rate compared with the controls (Table 2) except in the 0.3 g ashwagandha/100 g fed group. FCR were significantly better than controls in all groups receiving ashwagandha supplemented diet.

**Table 2:** Growth parameters of *L. rohita* fed with diet supplemented with ashwagandha.

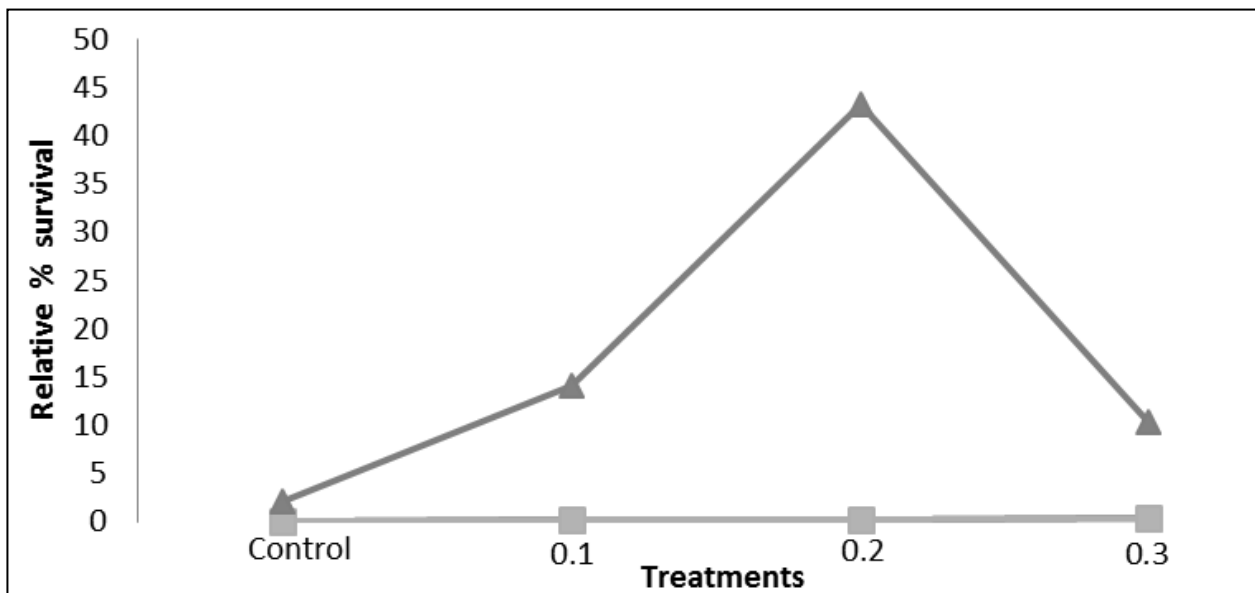
Gash wag and ha/100g of feed	% weight gain	SGR	FCR
0	12.26±1.5	0.27±0.01	0.4±0.0
0.1	16.33±0.7*	0.36±0.01*	0.1±0.2*
0.2	22.84±0.6*	0.48±0.02*	0.1±0.1*
0.3	14.32±0.4	0.31±0.01	0.2±0.5*

Data expressed as mean ± SE, n=10.

SGR: specific growth rate; FCR: feed conversion ratio

\*Significantly different from controls ( $p<0.05$ ).

**3.2 Relative percentage survival:** The relative survival percentages of *L. rohita* fingerlings in different experimental groups after challenge with *A. hydrophila* are shown in Fig.1. The highest RPS was recorded in the 0.2 g of ashwagandha/100 g of feed group (43.00±0.75%) and the lowest PRS was observed in 0.3 g ashwagandha/100 g fed group (10.00±0.02%).



**Fig 1:** Relative percentage survivals of *Labeo rohita* fingerlings following an experimental *Aeromonas hydrophila* infection.

**3.3 Haematological parameters:** The hemoglobin content, total leucocytes was significantly ( $p<0.05$ ) higher in all the groups that received ashwagandha compared to the controls (Table 3). Total erythrocytes was significantly ( $p<0.05$ ) higher in 0.2 g of ashwagandha/100 g of feed compared to the controls and haematocrit value was significantly ( $p<0.05$ ) higher in 0.2 g and 0.3g of ashwagandha/100 g of feed compared to the control. Also there was a significant change in the relative proportions of lymphocytes, monocytes and neutrophils, but not thrombocytes following feeding with

ashwagandha (Table 3). Thus there were a higher proportion of lymphocytes compared with controls in the group fed 0.1 g and 0.2 g of ashwagandha/100 g of feed but a lower proportion in the 0.3 g ashwagandha/100 g fed group. The proportion of neutrophils and monocytes was significantly higher in all groups with ashwagandha in the diet. The proportion of monocytes was virtually doubled in all ashwagandha fed group except for the group fed 0.3 g ashwagandha/100 g fed group. Eosinophils and basophils were not observed in any of the blood sample.

**Table 3:** Haematological parameters for *Labeo rohita* fed with different concentrations of ashwagandha for 42 days.

Gash wag and ha/10 g of feed	RBC ( $\times 10^6$ /mm <sup>3</sup> )	WBC ( $\times 10^3$ /mm <sup>3</sup> )	Hb (g/dL)	Hct (%)	Proportion (%)			
					Lymphocytes	Monocytes	Neutrophils	Thrombocytes
0	0.75±0.33	29.2±0.24	1.5±0.2	28.6±0.3	31.2±0.3	13.3±0.2	34.1±0.5	14.3±0.7
0.1	0.78±0.39	64.7±0.48*	3.5±0.1*	28.3±0.8	33.3±0.2*	24.2±1.2*	38.9±1.0*	7.3±0.9
0.2	1.05±0.04*	74.3±0.24*	5.08±0.0*	32.0±0.5*	35.1±0.7*	27.3±1.4*	39.4±1.1*	6.7±0.8
0.3	0.73±0.08	45.4±0.03*	3.53±0.0*	31.3±0.6*	31.5±0.1	18.2±1.1*	37.4±1.3*	6.9±0.7

Date expressed as mean ± SE.

RBC: Erythrocytes; WBC: White Blood Cells; Hct: Haematocrit value; Hb: Haemoglobin.

\*Significantly different from controls ( $p < 0.05$ ).

**3.4 Biochemical parameters:** The serum total protein content was significantly different from the controls in 0.2 g ashwagandha/100 g fed group only. The highest protein content was found in 0.2 g of ashwagandha/100 g of feed group (Table 4). Furthermore, all feeding levels of ashwagandha resulted in a significant increase in globulin content (Table 4). There were no significant differences in

serum albumin content (Table 4). A/G ratio showed a significant effect of ashwagandha in the experimental groups (Table 4). The blood glucose level was significantly higher in 0.1 and 0.2 g ashwagandha/100 g fed group. There were no significance differences in serum ALT activity, serum AST activity, Alkaline Phosphatase level of fishes compared to control, (Table 4).

**Table 4:** Biochemical indices of *Labeo rohita* after feeding with different concentrations of ashwagandha for 42 days.

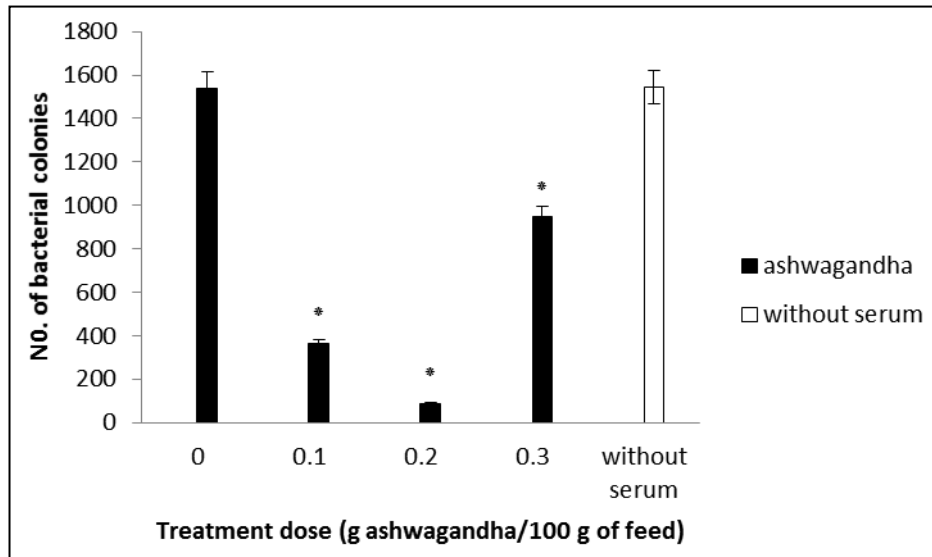
Gash wag and ha/10 g of feed	Total protein (g/dL)	Albumin (g/dL)	Globulin (g/dL)	A/G ratio	Blood glucose (g/dL)	AST (u/mL)	ALT (u/mL)	Alkaline Phosphatase activity (KA unit)
0	5.75±0.61	1.23±0.04	1.28±0.04	0.96±0.04	43.07±3.85	310±0.16	113±0.16	9.02±0.04
0.1	5.91±0.29	1.22±0.04	1.85±0.05*	0.51±0.01*	52.07±2.37*	297±0.16	110±0.20	6.5±0.33
0.2	8.30±0.79*	1.23±0.00	1.98±0.03*	0.48±0.01*	74.95±2.07*	286±0.57	108±1.58	5.55±0.04
0.3	6.89±0.09	1.21±0.07	1.78±0.02*	0.60±0.03*	51.14±0.60	289±0.16	112±0.80	4.55±0.05

Date expressed as mean ± SE.

\*Significantly different from controls ( $p < 0.05$ ).

**3.5 Serum bactericidal activity:** Serum bactericidal activity was significant in all ashwagandha fed group compared with the control. The viable bacterial colony counts were

significantly lower in these groups compared with the controls (Fig. 2).



**Fig 2:** Effect of feeding ashwagandha in the diet on the serum bactericidal activity of *Labeo rohita*. Values expressed as mean ± SE; \*significantly different from controls ( $p < 0.05$ ,  $n = 10$ ).

#### 4. Discussion

The results of this study reinforce the growing view that some plants are beneficial to fish by conferring protection against disease and stimulating the immune response [3].

##### 4.1 Growth performance and relative percentage survival:

In the present study, feeding ashwagandha to *L. rohita* led to enhanced growth are in line with cultured greasy grouper *Epinephelus tauvina* feeding with 100 and 200 mg of ashwagandha/kg of feed improved weight gain, SGR and FCR

[7]. Presence of certain steroids like ecdysteroids (20-hydroxyecdysone) in the plant extracts is reported to promote growth, like many other steroid hormones, by enhancing the feed conversion efficiency [35], this may be the possible reason for higher growth observed in the present study as ashwagandha has been reported to contain 35 chemical constituents, most of them being steroids and alkaloids [36]. The disease control potential of ashwagandha is certainly in the present study are supported from previous work [7], which noted the effectiveness of purified active principle of

ashwagandha/kg of feed for controlling *Vibrio harveyi* infection in cultured greasy grouper *E. tauvina* and *Achyranthes aspera* led to reduced mortalities in *Labeo rohita* [34].

**4.2 Serum bactericidal activity and Biochemical parameters:** The evidence from the present study is that the mode of action reflected enhancement of the immune response, which is in line with previous studies Sivaram *et al.* [7], who reported significant improvements in immune parameters such as serum bactericidal activity, serum globulin level and Albumin–globulin (A/G) ratio following feeding dietary ashwagandha to greasy grouper *E. tauvina*. The increases in serum total protein and globulin contents, as recorded in this study, are considered to reflect strong innate immunity [37]. Furthermore the data for the serum bactericidal activity indicate enhancement of process in the killing and clearing of pathogenic organisms in fish [38]. In this study, the highest protein and globulin concentration was found in the 0.2 g of ashwagandha/100 g of feed group. Lower albumin-globulin ratio indicates the presence of more amounts of globulin and in the present study lowest A/G ratio was found in 0.2 g of ashwagandha/100 g of feed group. Since the gamma fraction makes the largest portion of globulin, it can be inferred that 0.2 g of ashwagandha/100 g of feed group may have enhanced the immune response of *L. rohita* fingerlings.

In the present study the blood glucose level was significantly higher in 0.1 and 0.2 g ashwagandha/100 g fed group indicates the handling stress results high demand of glucose by tissues of fish in response triggering muscle or liver glycogenolysis and releasing glucose for the increased energy demands during and after stress [39]. The study of different enzyme activities such as serum ALT, AST, ALP has great value in the assessment of clinical and experimental liver damage [40]. In the present study insignificant ALT, AST, ALP level can be substantiated by the tendency of ashwagandha to return of these marker enzymes to near-normalcy [41].

**4.3 Haematological parameters:** The enhancement of leucocytes, erythrocytes, hemoglobin content and haematocrit value with the supplementation of ashwagandha in the diet of the present study indicating an improvement in the health status of the fish [27]. Indeed the results of this study support the work that dietary ashwagandha increased leucocyte count, erythrocyte count, haemoglobin content in mice and haematocrit value in human [42]. WBC is known as first line of defence [43] and play a major role in innate immunity and increase in their numbers in the present study indicate the role of ashwagandha in innate immunity. The use of ashwagandha in preventing disease may be related to its effect on the immune system.

A possible mode of action of ashwagandha in immunostimulation as a results of its bio active constituent Withaferin A (glycowithanolides) and a mixture of sitoindosides IX and X which play a role in immunostimulation revealed by the elicitation and activation of macrophages present in the blood stream through receptor expression [44]. A significant increase in lymphocytes produced a significant increase in the number and activation of CD4 cells results in increased of receptor expression (surface Fc receptor) indicating a major change in immune cell activation in fish [45]. The surface Fc receptor binds the Fc fraction of the antibody. Through the Fc receptors the

peritoneal macrophages can bind the target-coated cells and the macrophages elicited by these compounds produce a significant number of super rosettes which results activation of macrophages and immunostimulation (<http://www.freepatentsonline.com>). As neutrophils from the major fraction of the phagocytic leucocytes, it can be concluded that ashwagandha may be responsible for the increased phagocytic activity. Also Withaferin A (glycowithanolides) in ashwagandha has been regarded to have potent antioxidant properties being an effective scavenger of superoxide radicals which has been proposed as possible protective mechanism against autotoxicity and lethality [46]. This result was further supported by the amplification of the non-specific immunological defence played by Lysosomal enzymes secreted by the activated macrophages and phagocytic activity in *L. rohita* [47].

## 5. Conclusion

The present study has clearly highlighted the potential value of ashwagandha at 0.2 g/100 g of feed enhanced the growth performance of fish and protection against specific bacterial pathogen in *L. rohita* due to the immunostimulation. It remains for further work to consider the potential of other plants and their products for use in disease control strategies.

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