

Immunomodulatory Effect of Triphala Biofortification in *Oreochromis mossambicus*

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Abstract: The present study was conducted to evaluate the bio fortification efficacy of methanolic extract of Triphala in *Oreochromis mossambicus* as reflected by haematological parameters. Haematological parameters like total erythrocyte count (TEC), total leucocyte count (TLC), differential leucocyte count (DLC), haemoglobin (Hb), hematocrit (Ht), mean cell haemoglobin (MCH) serum total protein, albumin and globulin were analyzed in Triphala (0.8%) fortified *O. mossambicus* subsequently challenged with *Aeromonas hydrophila*. Significant increase in TEC, TLC, Hb, Ht, MCH and serum total protein and albumin were observed in Triphala supplementation group compared to control and increase in globulin level was insignificant. Post *Aeromonas hydrophila* infection, TLC, DLC and survival status improved in Triphala fortified group compared to control. Hence present study confirms that Triphala (0.8%), supplementation in fish feed acts as an immunomodulator.

Keywords: *Oreochromis mossambicus*, *Aeromonas hydrophila*, haematology, Immunostimulants

1. Introduction

Population explosion urges intensive aquaculture and development of new species-specific fish feed formulations supports the intensive aquaculture industry. Disease management through antibiotics and chemicals lead to bacterial resistance and accumulation of unacceptable residues in aquaculture products and environment which will be a threat to the health of consumers (5, 13, 6). Hence consumer and eco-friendly disease preventive measures to promote sustainable aquaculture is the call of the century. It is proved beyond doubt that medicinal herb incorporated aqua feeds provide basic nutritional requirements to improve growth, feed utilization and also the support of general health and stress resistance. Phytochemicals offer a very complex blend of bioactive components. Hence numerous research works progresses globally to standardize useful plant materials as feed additives to fin fish and shellfishes due to its multifunctional properties derived from their specific bioactive components, so that sustainable intensive aquaculture system can be streamlined. (3,7). The antibacterial activity of *Centella asiatica* and *Curcuma longa* against pathogenic bacteria affecting *Macrobrachium rosenbergii* was reported by Salini, et.al. (2015). Methanolic extract of *Curcuma amada* effectively control both opportunistic and pathogenic bacteria in *Oreochromis mossambicus* (14). Globally people have understood the malicious effects of antibiotics, and they are now shifting over to natural products.

Blood biochemistry has long been a supportive diagnostic tool in pathological, toxicological and general clinical tests (11). Haematological studies can be effectively used to prevent the occurrence of epidemics and thus to reduce economic loss to aqua farmers. Triphala, is a herbal formula prepared by mixing the dried powder of *Emblica officinalis*, *Terminalia chebula*, and *Terminalia bellerica* (1:1:1 proportion). Triphala purifies blood, stimulates bile secretion and detoxifies the liver. It helps in the digestion and assimilation, lowers serum cholesterol and lipid levels the

body, stimulate immune system and reduces high blood pressure. It has antioxidant, antimutagenic, antineoplastic, chemo protective, radio protective, and chemo preventive properties and also effective against cancer (4).

Tilapia is an important model to study fish physiology, particularly because of its broad tolerance to an array of environments. Currently Tilapia are the second most farmed fish in the world with an annual production exceeding 2.8 million tons in 2010 and they exhibit a versatile adaptability to different environmental conditions to match the vast array of their ecological habitats.

Hence the objective of the present study is to assess the bio fortification efficacy of Triphala in *O. mossambicus* with emphasis on immunomodulatory potentials as reflected by haematological parameters.

2. Materials and Methods

2.1. Experimental set up

The fruits of medicinal plants, *Emblica officinalis*, *Terminalia bellerica* and *Terminalia chebula*, were collected and identified in Pankajakasturi Ayurvedic Research Centre, Thiruvananthapuram. *O. mossambicus* having 30-40 gm wt. were collected from Agency for Development of Aquaculture in Kerala (ADAK) at Varkkala, quarantined and stocked in 1000 L glass tanks, and maintained at normal laboratory conditions. After two weeks acclimatization the fish are randomized into two groups, one group served as control and was supplemented with control feed and the other group was fed with medicated feed (D1=0.8% Triphala) prepared in the laboratory.. Both of them were fed @ 2% of their body weight twice daily. Water quality parameters were maintained every day 50% water was renewed daily during removal of fecal materials and unused feed. During the experimental period the water quality variables: temperature (28±1°C), pH (7.2±0.2), and dissolved Oxygen (7.5±0.5) mg/l were recorded. The

experimental regime continued, in triplicate for two months and hematological parameters pre and post *A. hydrophila* challenge were assessed and documented.

Blood samples were collected using 1 ml syringe directly from the ventral aorta and transferred into sterilized glass vials containing one milligram of EDTA and was used to estimate total RBC, WBC, DLC and Hb, and haematocrit (1). Blood without anticoagulant was used to estimate serum total protein and albumin levels. Plasma was obtained by centrifugation of blood at 3000 rpm for 15 min and was stored in deep freezer for further biochemical analysis. Total Erythrocyte Counts (TEC), Total Leucocyte Counts (TLC) were counted using Haemocytometer with improved Neubauer chamber (Weber & sons, England), Haemoglobin was measured using cyanohaemoglobin method using Drabkins fluid. Blood smears stained with May-Grünwald's Giemsa's stain was used for differential leucocytes count.

2.2 Methanolic extract preparation

The methanolic extract preparation was done as outlined by Singh, (2008) incorporating modification of Jasmine *et al.* (2011). Fresh fruits of *Emblica officinalis*, *Terminalia chebula*, and *Terminalia bellerica*, were washed in sterilized water, shade dried, (de seeded) and grinded separately. Fruit powder was successively extracted with 85% absolute methanol and then subjected to vacuum filtration. The filtrate is dried and the residue obtained after evaporation was carefully collected, weighed and transferred into air tight containers and stored at -20°C till use.

2.3 Feed preparation

The control diet was prepared by mixing fish meal 40gm, rice brawn 25gm, ground nut oil cake 10 gm, soya 10 gm, tapioca powder 5 gm, wheat flour 5 gm, with 100 ml sterilized water and cooked in pressure cooker for 5 minutes. Cooked feed is allowed to cool and fish oil 2 ml, vegetable oil 2 ml, and one vitamin & mineral mix tablet was added. After proper mixing it is squeezed through a pelletizer, and shade dried. For experimental diet, 0.8% methanolic extract of Triphala was added to the control feed before pelletizing.

2.4 Challenge study

Subsequent to the feeding regime (60 days) both control and experimental fishes were challenged intra peritoneally with *Aeromonas hydrophila* (10⁸ cfu/ml) and 7 days post challenge fishes were subjected to hematological studies.

2.5 Statistical Analysis

The statistical analysis was carried out using the software SPSS 17.0 package. For determining the significant difference between different treatments in blood and serum parameters one-way ANOVA followed by Duncan's test was done. Significance level (P value) was set at 0.05 in all tests. Values in the same row with different lower case letters vary significantly (p<0.05) between treatment groups. Each value represents the mean ± SE of six separate data.

3. Results and Discussion

Proximate analysis of the control and experimental feed is given in Table 1. The results obtained as per the experimental design are given in Tables 2-6.

Table 1: Proximate composition of feed

In 100 gm feed (gm %)	Control feed	0.8% Triphala
Protein	35.41 ± 0.08 ^a	35.20 ± 0.04 ^a
Carbohydrate	20.12 ± 0.01 ^a	20.21 ± 0.01 ^a
Lipid	10.18 ± 0.18 ^a	10.15 ± 0.17 ^a
Ash	12.84 ± 0.22 ^a	12.55 ± 0.12 ^a
Moisture	10.05 ± 0.21 ^a	10.14 ± 0.09 ^a
Dry matter	89.95 ± 0.52 ^a	89.86 ± 0.35 ^a
Fiber	4.39 ± 0.09 ^a	4.58 ± 0.06 ^a
NFE %	37.18 ± 0.11 ^a	37.52 ± 0.08 ^a
GE	448.5 ± 0.01 ^a	448.5 ± 0.01 ^a

In proximate analysis of feed no significant variation observed in any of the components assessed. Highly significant increase in total erythrocyte count (TEC) Hb, Haematocrit, MCV and MCH in Triphala fortified group compared to control. (Tables 2). Increased levels of TEC, TLC and serum protein in Triphala supplemented group is an indicator of improved general health (Tables 3). In the experimental groups the lymphocyte were higher followed by neutrophil, polymorphs and, eosinophil. The lymphocytes exhibited an increasing trend in D1 (0.8% Triphala) fed group post challenge it presents the immune readiness of the treated group as both non-specific and specific immune response depends upon the population of leucocytes. Sahu *et al.*, (2007a) reported an enhanced WBC & RBC counts in *Labeo rohita* fingerlings supplemented with *Mangifera indica* kernel. Prit Benny *et al.*, (2010) also noted an increase in WBC and lymphocyte counts in *Clarias batrachus* fed with *Musa acuminata* peel extract. Such a rise in RBC was also observed by Mohsen Abdel-Tawwab *et al.*, (2010) in Nile Tilapia fed with green tea. Maintenance of steady population of neutrophils and monocytes is vital in resisting general infection and to induce the specific immunity by increasing the lymphocytes. Post challenged herbal supplemented fishes quickly reestablished the altered haematological and serological parameters.

Triphala supplemented group showed significant increase in serum glucose level may be due to an increased cortisol level produced by bacterial infection (Tables 4&5). Above results indicate that oral administration of the methanolic extract of Triphala (0.8%) is very effective in elevating the levels of hemoglobin MCV, MCH and haematocrit in the erythrocyte of fish. Triphala is supposed to increase digestion and appetite, and very effective in detoxification of toxic substances. Reduction in TEC coupled with low Hb content after challenge with *Aeromonas hydrophila* in control group may be due to pathological condition. In our study TLC increased in Triphala supplemented fishes and 87 % of herbal treated group survived without any outward manifestation of symptoms even after 7 days of post challenge. This may be due to specific immune response developed in fish due to bio fortification effect of Triphala.

Significant decrease in RBC's hemoglobin and packed cell volume subsequent to challenge in control *O. mossambicus*

shows that *Aeromonas* infection had affected both metabolic and hematopoietic activities of control tilapia. Similar results were also observed by Sudagar and Hajibeglou (2010) in *C. carpio* supplemented plant extracts for 60 days and challenged with *A. hydrophila*. Increase in serum total protein and albumin levels in D-I diet fed present work may be an indication to increased levels of non-specific immunity and the increased albumin levels may facilitate the transport of more humoral compounds as well as active principles present in the herbal extract through the blood. The haemopoietic expansion of innate immune cells could be reflective of an elevated state of immuno-readiness. Thus from the present study it was evident that incorporation of Triphala, in fish feeds helps to improve the general health and also to recover from biological stress often encountered by fishes in their natural habitat

Table 2: Total RBC, Hb, Haematocrit, MCV, MCH and MCHC values of *Oreochromis mossambicus* reared on Triphala supplemented diet and challenged with *Aeromonas hydrophila*.

Hematological parameters	Pre challenge		challenged	
	Control	D-I	Control	D-I
Total RBC ($10^6/\text{mm}^3$)	2.2±.097 ^a	2.8±.064 ^b	1.85±.031 ^a	2.18±0.05 ^b
Hb (g/dl)	5.60±.124 ^a	8.0±.045 ^b	4.22±0.08 ^a	7.14±0.06 ^b
Hct (%)	25±.042 ^a	35±.044 ^b	20.04±0.41 ^a	30.2±0.31 ^b

Table 4: Serum protein parameters in Triphala bio fortified *Oreochromis mossambicus*

Test Diets	Serum protein parameters							
	Pre challenge				Post Challenge			
	Total protein (g/dl)	Albumin (g/dl)	Globulin (g/dl)	A/G ratio	Total protein (g/dl)	Albumin (g/dl)	Globulin (g/dl)	A/G ratio
Control	2.94±.050 ^a	1.05±.045 ^a	1.89±.259 ^a	0.55±.003 ^a	3.15±.056 ^a	1.43±.038 ^a	1.72±.075 ^a	0.83±.009 ^a
D-I	3.95±.035 ^b	1.87±.115 ^b	2.08±.097 ^b	0.89±.002 ^b	4.15±.061 ^b	2.0±.109 ^b	2.15±.026 ^b	0.93±.024 ^b

Serum protein parameters presented a statistically significant increase in Triphala supplementation groups (D-I) compared to control. Subsequent to challenge globulin presented a statistically significant increase in Triphala fortified fishes compared to control.

Table 5: Plasma glucose level in Triphala bio fortified *Oreochromis mossambicus*

Test Diets	Plasma Glucose mg/dl	
	Pre challenge	Challenged
Control	86±2.77 ^a	132±1.15 ^b
D-I	82±1.71 ^a	109±5.65 ^a

Plasma glucose level in pre challenged *O. mossambicus* is not statistically significant but in challenged fish the plasma glucose level significantly increased in control.

Table 6: Percentage of survival in Triphala bio fortified *Oreochromis mossambicus*

Test Diets	survival Percentage	
	Pre challenge	Challenged
Control	96.6±1.02 ^a	53.3±1.15 ^a
D-I	100.0±0.00 ^b	86.6±5.65 ^b

Statistically significant increase in survival percentage was observed in herbal fortified prechallenged and challenged fish.

MCV μm^3	113.6±1.24 ^a	125±1.15 ^b	108.1±0.67 ^a	138.5±1.09 ^b
MCH pg.	25.45±0.62 ^a	28.5±0.28 ^b	25.45±0.82 ^a	32.76±0.48 ^b
MCHC (%)	22.4±0.42 ^a	22.86±0.45 ^a	21.06±0.62 ^a	23.65±0.59 ^b

RBC, Hb, Haematocrit, MCV, and MCH values of *O. mossambicus* supplemented with Triphala is statistically significant in D-I from control, MCHC values are not statistically significant from control. All parameters are statistically significant in D-I in challenged fishes.

Table 3: Total and differential leucocyte count in Triphala bio fortified *Oreochromis mossambicus*

Hematological parameters	Pre challenge		Challenged	
	Control	D-I	Control	D-I
TLC ($\times 10^3$)	8±0.28 ^a	8.5±0.12 ^b	6.569±.281 ^a	9.039±.5.97 ^b
Lymphocyte (%)	75±1.48 ^b	73±1.11 ^a	55.01±1.77 ^a	84.11±.148 ^b
Polymorphs (%)	10.5±0.37 ^a	17±0.39 ^b	28.7±.378 ^b	15.82±.981 ^b
Neutrophil (%)	14.45±0.12 ^b	9.97±0.43 ^a	15.28±.378 ^b	0.07±.128 ^a
Eosinophil (%)	0.04±0.01 ^a	0.02±0.01 ^a	1.0±.129 ^b	0.08±.003 ^a
Monocytes	0.01±0.01 ^a	0.01±0.01 ^a	0.02±.002 ^a	0.03±.004 ^a

Total and differential leucocyte count in *O. mossambicus* presented a statistically significant increase in Triphala supplementation groups compared to control. But in challenged fishes control values fall beyond normal.

Serum Biochemical Parameters

4. Conclusion

The poly factorial phytonutrient cocktail present in *Triphala* had significantly improved haematological and serological parameters. The inclusion of herbal additives in diets often provides cooperative action to various physiological functions. The haemopoietic expansion of innate immune cells could be reflective of an elevated state of immuno-readiness. The present hematological data provides valuable information in assessing the health of *O. mossambicus* and in monitoring stress responses to *Aeromonas* infection. Present study can be concluded by stating that methanolic extract of (*Triphala* 0.8%) is quiet effective as an immunomodulator. This preliminary work attempts to say little about the physiological mode of action of the plant extract in *Oreochromis mossambicus* but surely is an active ecofriendly area for future research work.

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