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Nutritional evaluation of heat-treated and raw leaf powder of *Medicago sativa* for freshwater fish, *Cirrhinus mrigala*

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Abstract
A 90 day feeding trial was conducted to assess the efficacy of *Medicago sativa* plant leaf powder as a supplementary feed ingredient for the fish *Cirrhinus mrigala*. Six experimental diets were formulated by replacing fishmeal from standard diet at the proportion of 30%, 40% and 50% by alfalfa leaf powder. Three of them kept as raw and remaining three subjected to hydrothermal processing. The weight gain, specific growth rate, food conversion efficiency, biochemical composition was not largely affected by the plant powder incorporated feed as compared to control feed containing rice bran and groundnut oilcake as main ingredients. This study revealed that the alfalfa plant powder may be a promising source to replace fishmeal up to 50% inclusion level.

Keywords: *Cirrhinus mrigala*, feed processing, formulated diet, leaf meals, *Medicago sativa*

1. Introduction

Nutrition holds a crucial role in the creation of robust and top-notch fish products. Recent times have witnessed significant progress in fish nutrition, particularly regarding the development of well-balanced commercial diets. These diets are instrumental in fostering the growth of sturdy and healthy fish. Among the array of nutritional sources available, fish meal stands out as a prime supplier of dietary protein for fish. With its remarkable biological value, it has secured its position as the foremost protein source in the aquaculture sector. The meticulous design of fish diets assumes a paramount importance in ensuring comprehensive fish nutrition [1, 2].

Fish meal has risen to prominence as the most expensive protein constituent within aquaculture dietary plans. This heightened expense, coupled with its limited availability, can pose challenges for regular fish farmers striving to integrate fish meal as the primary protein origin in their aqua feeds. In response, fish nutritionists have undertaken efforts to tackle this issue by partially or fully substituting fish meal with more economical and unconventional sources of animal or plant protein. Extensive research endeavors have showcased significant strides in partially replacing fish meal with plant-based components in diets tailored for a variety of fish species. Diligent attempts persist among nutritionists to curtail feed expenses as a means of fostering sustainable aquaculture practices. To overcome the financial burden posed by fish meal, the inclusion of locally accessible agro-based byproducts has become imperative in devising cost-effective and well-balanced fish diets. Noteworthy research attention has been directed towards various plant-derived meals as potential protein reservoirs in animal nutrition [3-5].

Nonetheless, substituting fish meal entirely with single plant proteins has typically led to a reduction in fish growth performance. This decline is often linked to the occurrence of anti-nutritional compounds in plants. However, these anti-nutritional factors within feed ingredients can be neutralized through methods such as hydrothermal processing and fermentation [6].

The alfalfa plant (*Medicago sativa*) has gained extensive usage in terrestrial animal diets, owed to its abundant protein content accompanied by a well-balanced amino acid composition, as well as vitamins and carotenoids.

However, incorporation of alfalfa-based products into fish feeds have yielded variable outcomes [7, 8]. The present study was conducted to assess the viability of incorporating alfalfa leaf meal into the diet of *Cirrhinus mrigala* and its effect on growth and biochemical constituent of fish.

2. Materials and Methods

2.1 Sample collection and processing

Fresh *Medicago sativa* leaves were gathered, carefully cleansed to eliminate soil and debris, drained, and subsequently air-dried. The dried leaves were then finely ground through a kitchen mixer, packaged in hermetic polyethylene bags, and stored in a refrigerator at approximately 20 °C until required. Six distinct diets were formulated, each comprising varying combinations of dry *M. sativa* leaf powder, groundnut oil cake, rice bran, binder (Guar gum), and a vitamin-mineral mixture powder (Table 1). Among these diets, three underwent heat cooking, while the remaining trio remained unprocessed. Additionally, a control diet was formulated, featuring groundnut oil cake, rice bran, and fish meal. The ingredients were diligently blended using a kitchen mixer until achieving a uniform mixture. This mixture was subsequently used to prepare pelletized feed using a mincer, followed by sun-drying of the pellets. Once dried, the pellets were manually broken down into suitable sizes and preserved in the refrigerator until just before feeding.

Table 1: Percentage of different ingredients used in Raw and Heat treated formulated pelleted diets (per 100 gm)

Ingredients	Control	30%	40%	50%
Rice bran	52	19	17	14
Groundnut oilcake	37	40	32	25
Guar gum Binder	10	10	10	10
Mineral – Vitamin mixture	01	01	01	01
Plant Powder	00	30	40	50

2.2 Experimental system and fish

Fingerlings of the *Cirrhinus mrigala*, ranging from 2.00 to 2.50 grams in weight, were employed for the study. These fishes were allowed to acclimate in glass tanks for a period of 15 days. The feeding trial took place within aquaria measuring 6’ × 2’ × 2’, spanning a duration of 90 days. The water in the aquaria underwent alteration every other day, while consistent aeration was maintained. Each individual aquarium was stocked with 10 fingerlings of consistent size and weight. The fish were provided with feed equivalent to 5% of their body weight, divided into two equal portions per day. At intervals of two weeks, 50% of the fish population was sampled for growth assessment purposes.

2.3 Analytical procedures and water quality assessment

The major feed ingredients and the formulated diets were analyzed for proximate composition as shown in table 2 and 3 in accordance with AOAC protocols [9]. The physicochemical analysis of water used during experimentation was carried out as described by APHA [10].

Table 2: Proximate composition of pelleted diets (per 100 gram) Raw Feed

Parameter	Control	30%	40%	50%
Moisture (%)	7.05	5.19	6.79	5.84
Total Ash (%)	12.13	9.39	9.48	9.54
Fat (%)	3.81	3.28	3.36	3.70
Crude Protein (%)	19.24	27.45	29.77	26.94
Crude Fiber (%)	16.54	10.05	10.85	12.51

Table 3: Proximate composition of pelleted diets (per 100 gram) heat treated feed

Parameter	Control	30%	40%	50%
Moisture (%)	7.05	5.86	7.61	6.18
Total Ash (%)	12.13	9.36	9.34	9.01
Fat (%)	3.81	3.18	3.54	3.23
Crude Protein (%)	19.24	26.58	28.68	24.67
Crude Fiber (%)	16.54	9.05	10.10	11.36

2.4 Diet performance evaluation

Growth performance of experimental fish was determined in terms of final individual fish weight (g), survival (%), Specific Growth Rate (SGR, % per day), food intake on a percentage of body weight basis, feed Conversion Ratio (FCR), Protein Efficiency Ratio (PER), Feed Efficiency Ratio (FER) and Net Protein Retention (NPR). These growth response traits were calculated as follows:

Weight gain (%) = 100 (final body weight - initial body weight).

SGR (% per day) = 100 (ln BWf – ln Bwi)/time.

FCR= [Dry weight of feed fed (g)]/ [Fish weight gain (g)].

PER= [Fish weight gain (g)]/[Protein fed (g)].

NPR = (Gain in carcass protein/ Weight of protein fed) ×100.

2.5 Analysis of fish tissue for biochemical study

Fishes were sacrificed at the beginning and at the end of the experiment to remove liver and muscle tissues. These tissues were used for the estimation of biochemical components such as protein [11], Glycogen [12] and lipids [13].

3. Results and Discussion

3.1 Growth performance

The growth performance of fish fed with various formulated diets containing different levels of *Medicago sativa* plant leaf powder meal is summarized in Table 4 and 5.

Table 4: Growth performance of fish with different experimental diets raw

	Control	30% Raw	40% Raw	50% Raw
Initial average length (cm)	4.05	4.18	4.13	4.15
Final average length (cm)	16.5	17.5	17.4	16.1
Initial average weight (gm)	2.15	2.4	2.2	2.2
Final average weight (gm)	12.6	13.7	13.3	12.1
Total live weight gain	10.45	11.3	11.1	9.9
Specific growth rate (SGR)	0.73	0.71	0.74	0.74
Food conversion ratio (FCR)	2.3	2.2	2.1	2.2
Protein efficiency ratio (PER)	2.0	2.1	2.3	1.9
Net protein retention (NPR)	22.08	22.76	25.19	22.27

Table 5: Growth performance of fish with different experimental diets heat treated

	Control	30%	40%	50%
Initial average length (cm)	4.05	4.15	4.15	4.15
Final average length (cm)	16.5	17.9	18.9	16.1
Initial average weight (gm)	2.15	2.3	2.1	2.2
Final average weight (gm)	12.6	14.9	16.4	12.1
Total live weight gain	10.45	12.6	14.3	9.9
Specific growth rate (SGR)	0.73	0.77	0.85	0.70
Food conversion ratio (FCR)	2.3	2.4	3.1	2.0
Protein efficiency ratio (PER)	2.0	2.5	2.8	2.2
Net protein retention (NPR)	22.08	26.33	30.50	23.30

The highest weight gain was seen in 40% heat treated group, while 50% raw showed least growth among all the feeds. All experimental groups showed better growth results as compared to control.

3.2 Biochemical alterations

The biochemical alterations in liver and muscle tissues of experimental fish fed with alfalfa meal was shown in table 6 and 7.

Table 6: Biochemical alterations in liver from the fish *C. mrigala* fed with *M. sativa* leaf meal

	Total Protein	Total Lipid	Total Glycogen
Control	17.53±0.37	17.19±0.56	0.9±0.04
30% R	17.49±0.88 NS	15.53±0.35 NS	1.63±0.21 **
40% R	18.59±0.49 NS	12.60±0.20 *	0.77±0.02 NS
50% R	11.83±0.57 ***	14.16±1.15 NS	0.65±0.05 NS
30% HT	13.36±0.48 **	13.29±1.27 *	0.67±0.10 NS
40% HT	21.24±0.41 **	11.23±0.52 **	0.82±0.02 NS
50% HT	12.43±0.36 ***	13.48±0.72 NS	0.62±0.02 NS

R: Raw; HT: Heat treated
(Value expressed in mg/100mg wet tissue;±: SE)
*$p<0.05$, **$p<0.01$, ***$p<0.001$, NS – Non Signifiant

Table 7: Biochemical alterations in muscle from the fish *C. mrigala* fed with *M. sativa* leaf meal

	Total Protein	Total Lipid	Total Glycogen
Control	22.34±0.40	6.16±0.14	0.16±0.01
30% R	32.36±0.78 ***	8.73±0.16 **	0.48±0.01 ***
40% R	35.16±0.54 ***	4.68±0.30 NS	0.47±0.05 ***
50% R	25.82±0.33 *	8.70±0.37 **	0.34±0.02 **
30% HT	39.25±0.14 ***	5.19±0.34 NS	0.44±0.01 ***
40% HT	44.31±1.15 ***	4.45±0.30 NS	0.70±0.01 ***
50% HT	22.31±0.76 NS	7.73±0.63 NS	0.31±0.01 *

R: Raw; HT: Heat treated
(Value expressed in mg/100mg wet tissue;±: SE)
*$p<0.05$, **$p<0.01$, ***$p<0.001$, NS – Non Signifiant

The 40% heat treated diet shows highest protein content in both liver ($p<0.01$) and muscle ($p<0.001$), whereas the 50% raw feed shows related lowest protein content. As compared with control diet, all plant protein based diets showed better protein content increase. The lipid content of liver and muscle was higher in control diet, and lowest in 40% heat treated diet. The glycogen content was very less in both the tissues.
In the present study, it is observed that the growth of fish was increased up to a certain limit depending upon the incorporation of plant protein in the feed. Above the optimum level of inclusion of plant proteins, the retarded growth was observed. In the present study, the experimental fish, *C. mrigala* showed good and increased growth up to 40% inclusion level and suddenly shows decreased growth for 50% inclusion. Among all diet groups, 40% heat treated feed showed highest growth and protein content, whereas raw feed showed comparatively low growth and protein content. Evaluating the palatability of a feed ingredient holds significance as a criterion when assessing its suitability for fish. The growth of fish is contingent upon both the ingredients used and their respective proportions within the formulated feed [2].
The growth of fish is influenced by the digestibility of a specific feed ingredient. The digestibility of a feed component hinges on factors like its inherent nature, dietary composition, nutrient type, and inclusion level. De Silva *et al.* [14] noted that the incorporation of plant ingredients in *Oreochromis aureus* led to a diminishing trend in protein digestibility as levels increased. In the current study, a gradual decrease in both NPR and PER values was observed with the progressive inclusion of raw *M. sativa* meal in the diets. Comparable downward trends in NPR and PER values have been previously reported when higher levels of plant protein were included [15, 16].
The feeding trials conducted on *C. mrigala* demonstrated a noteworthy increase in weight gain and notable biochemical changes in the fish groups that were provided with heat-treated feed, as compared to those fed on raw feed. These findings align with numerous other investigations that suggest heat treatment effectively diminishes anti-nutritional components present in plant protein diets. Moreover, the quality and acceptability of feeds are also influenced by heat treatment or cooking [17, 18]. Notably, hydrothermal processing additionally contributed to enhanced FCR values, exhibiting a connection with growth performance. The disparities in growth parameters and FCR values observed between raw and heat-treated feeds can be attributed to the deactivation of anti-nutritional factors resulting from the hydrothermal processing of the feeds [26].
At the conclusion of the feeding trial, the proximate carcass composition of the fish showed a significant increase in protein and lipid levels when compared to their initial values across all dietary treatments. The fish fed diets containing 40% heat-treated plant meal exhibited elevated values of carcass protein and lipid content. These findings align with earlier studies demonstrating similar trends with increased levels of fermented sesame seed, leaf meals, and grass pea seed meals in carp diets [20, 21]. The observed trends in carcass composition in this study are consistent with analogous outcomes reported in diets incorporating aquatic plants for Common carp [22], Moringa leaf meal for tilapia [23], alfalfa and aquatic plants for Nile tilapia [24], and heat-treated soybean meal for hybrid striped bass [25].

4. Conclusion

The current study affirms that *Cirrhinus mrigala* can effectively utilize a formulated diet primarily based on plant ingredients. Incorporating these plant materials at levels of up to 40% within the practical diet for *C. mrigala* fingerlings demonstrated no detrimental impacts on growth, feed utilization efficiency, or the fish's body composition. Notably, fish growth was notably more pronounced in response to the heat-treated feed compared to the raw variant. This enhanced growth might be attributed to the reduction of anti-nutritional components through hydrothermal processing of the plant leaf powder. In summary, this study underscores the potential of *Medicago sativa* as a promising source of plant protein, suitable for partially substituting fishmeal in formulated feeds.

This could significantly assist small-scale fish farmers in managing their expenditure on conventional fish feed products.

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