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HPLC screening of medicinal plant extracts and medicated fish feed for *Carrasius auratus* (Linn)

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Abstract
Aqueous extracts of selected medicinal plants such as *Leucas aspera*, *Achyranthes splendens,* and *Swertia chirayita* were prepared and screened for its protein content by High Performance Liquid Chromatography. With this background the present study aims at increasing the survival rate of ornamental fish. The self-formulated fish feed made from the extracts of *Leucas aspera*, *Achyranthes splendens,* and *Swertia chirayita* leaves and fishery wastes. The medicinal feed surpassed the quality of commercial fish feed by having higher protein and fibre along with medicinal properties. This enabled the fish to combat commonly found fish pathogens in water thereby increasing the survival rate.

Keywords: fishery wastes. medicated fish feed, protein, HPLC

1. Introduction
Formulation and preparation of fish feed is the processes of combining feed ingredients to form mixture that will meet the specific goals of production. It is often a compromise between an ideal formula and practical considerations. While formulating the feed one must take into account some considerations such as price, availability of ingredients used anti-nutritional factors and palatability of mixtures (Azevedo 1998; Windsor, 2001) [10]. Hence with this background the present investigation was focused on the formulation of medicated fish feed for the golden fishes in aquarium.
The self-formulated fish feed made from the extracts of *Leucas aspera*, *Achyranthes splendens,* and *Swertia chirayita* leaves and fishery wastes. The medicinal feed surpassed the quality of commercial fish feed by having higher protein and fibre along with medicinal properties. This enabled the fish to combat commonly found fish pathogens in water thereby increasing the survival rate and health of fishes.
Hence in the present investigation selected medicinal plants were used along with the fishery wastes to prepare the pellets. HPLC technique was used to study their secondary metabolites as medicated proteins. Weight of the golden fishes were assessed once in a week for their growth.

2. Materials and Methods
2.1. Preparation of Phyto Extracts
Aqueous extracts of selected plants such as *Leucas aspera*, *Achyranthes splendens,* and *Swertia chirayita* were prepared at 1:1 w/v.

2.2. Protein Extraction Using HPLC
1g of sample was weighed and macerated in pestle and mortar with 5 ml of phosphate buffer, and the contents were transferred to a centrifuge tube. The tube was centrifuged at 8000 rpm for 20 minutes. Supernatent was collected and the extraction was repeated 4-5 times. Supernatent was combined and the volume was made to 50 ml with phosphate buffer. To 5 ml of supernatant, 5 ml of 20% TCA was added and incubated for half an hour, and centrifuged at 8000 rpm for 20 minutes. Supernatant was discarded, and the pellet was washed twice with acetone.5 ml of 0.1 N NaOH was added to the pellet and mixed well to dissolve the pellet.

1. Ion Exchange Seperation
Ion exchange separation is based on the binding of charged sample molecules to oppositely charged groups attached to an insoluble matrix. Substances are bound to ion exchangers when they carry a net charge opposite to that of ion exchanger.

This binding is electrostatic and reversible.

2. Selection of Ion Exchanger
The pH value at which a bio-molecule carries no net charge is called the iso-electric point (pI). When exposed to a pH below its pI, the bio-molecule will carry a net positive charge and will bind to a cationic exchanger. At pH above its pI, the bio-molecule will carry a net negative charge and will bind to an anionic exchanger.

3. Selection of Buffer pH and Ionic Strength
Buffer pH and ionic strength are critical for binding and elution of material in ion exchange chromatography. Selection of appropriate pH and ionic strength for the start and elution buffers allows the use of 2 possible separation strategies. In the first strategy, binding is achieved by choosing a start buffer with a low pH for CM Sepharose and high pH for Q Sepharose Fast Flow. The ionic strength should be kept as low as possible to allow all components to bind to the ion exchanger. This results in concentration of the target substance and a complete picture of the total sample. In the second strategy, enrichment of target protein is achieved by choosing a start buffer with a pH optimized to allow maximum binding of target protein, and as high possible as ionic strength to suppress binding of sample contaminants. This strategy results in a concentration of the target substances.

4. Choice of Gradient
Continuous salt gradients are the most frequently used type of elution. Two buffers of different ionic strength, the start and the elution buffer are mixed together and if the volume ratio is changed linearly, the ionic strength changes linearly.

5. Sample Protein Purification
Buffers used

1. Start buffer: Tris-HCl (pH-9.0): 100 ml of 0.2 M Tris + 10 ml of 0.2 M HCl diluted to 400 ml of distilled water gives Tris buffer of pH 9.0.
2. Elution buffer: Tris-NaCl (pH-8.5): 150 ml of Tris-HCl (start buffer) + 150 ml of 2M NaCl gives Elution buffer.

Samples: Crude protein extracts of medicinal plants *L. aspera, A.splendens, S. chirayita.*
Initially the column was washed off the preservatives with 5 ml of 20% ethanol. The column was washed again with 5 ml of elution buffer. It was equilibrated with 5-10 ml of start buffer. Sample was applied at a flow rate of 1 ml/min using a syringe. The column was washed again with 5 ml of start buffer until no material appears in the effluent. The sample was eluted with 5-10 ml of elution buffer. Continuous gradient elution was performed and the pure protein fractions were collected. After the completed elution, the column was regenerated by washing with 5 ml of elution buffer followed by 5-10 ml of start buffer.

2.3. Protein Estimation (Lowry *et. al.,* 1946)
It is the most commonly used method for the determination of proteins in cell free extracts because of its high sensitivity, and quantities as low as 20 µg of protein can be measured. Suitable aliquots (1 ml) of the extracts were taken and to them, 4.5 ml of ml freshly prepared alkaline copper sulphate reagent was added. Folin's reagent was added and the contents were mixed instantaneously. The color was allowed to develop for 10 minutes. The absorbance was recorded at 660 nm after setting the spectrometer with reagent blank containing 1 ml of 0.1 N NaOH, instead of sample aliquot. In another set of test tubes, suitable aliquots of BSA solution (0-100 µg range) were taken. The total volume was made to 2 ml with 0.1 N NaOH and the color was developed as before. A standard graph of absorbance at 660 nm verses µg of BSA was plotted. From the standard graph, the amount of the protein present in the samples was determined. (Galley *et al.*, 2013)

2.4. Preparation of Medicated Feed Pellets
Fresh leaves of *Leucas aspera*, *Achyranthes splendens,* and *Swertia chirayita* were collected from the college campus, air dried for 2 -3 days and powdered. The dried leaves were ground to a fine powder in a mixer. The dried powder was taken in the following order and proportion (W/W).
Leucas aspera: *Achyranthes splendens: Swertia chirayita* (LAS)

- 1:1:1(LAS)
- 0.5:1:1 (LAS)
- 1:0.5: 1(LAS)
- 1:1:0.5 (LAS)

Along with 50% fishery wastes the medicated mix was bound into a thick dough using the gel of Aloe vera as binding agent. The dough was then made into fine uniform pellets which were eventually air dried.

2.5. Test on Weight of Carrasius *Auratus* Fishes
The test was conducted with five treatments including the control in triplicates of Completely Randomized Block Design. Every week weight of *Carrasius auratus* was observed and recorded for various treatments (T1 to T5)
T1- control-Jet X –fish food
T2 - 1:1:1 (LAS) + 50% fishery wastes
T3 - 0.5:1:1 (LAS) + 50% fishery wastes
T4 - 1:0.5: 1 (LAS) + 50% fishery wastes
T5 - 1:1:0.5 (LAS) + 50% fishery wastes
The golden fishes were weighed and results were recorded once in a week upto four weeks.

3. Results and discussion
3.1. Protein Profile of different sources of fish feed
The qualitative study of the proteins of the phyto extracts was performed by High Performance Liquid Chromatography. The resulting chromatograms were recorded and presented in the Figure 1, 2 and 3. The analysis was supportive to the quantitative analysis. The protein content of the medicinal plants were purified and taken as pellets along with the fishery wastes.
The present study confirms the quality of protein in the fish feed (Table-1) because many fish farming practices concern about the quality of protein. Commercial protein Jet-X was used to compare the protein profile of fish feed.
Similar studies of Webster *et al.*, 1992 [9]; Bai and Gatlin, 1992 revealed that protein is used for fish growth if adequate levels of fats and carbohydrates are present in the diet. If not, protein may be used for energy and life support rather than growth. Proteins are composed of carbon (50%), nitrogen (16%), oxygen (21.5%), and hydrogen (6.5%). Fish are capable of using a high protein diet, but as much as 65% of the protein may be lost to the environment. Most nitrogen is excreted as ammonia (NH_3) by the gills of fish, and only 10% is lost as solid wastes.

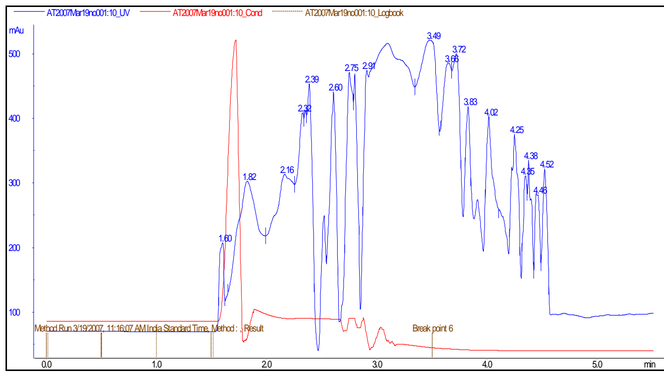

Fig 1: HPLC Chromatogram of Protein Profile of *L. aspera*:

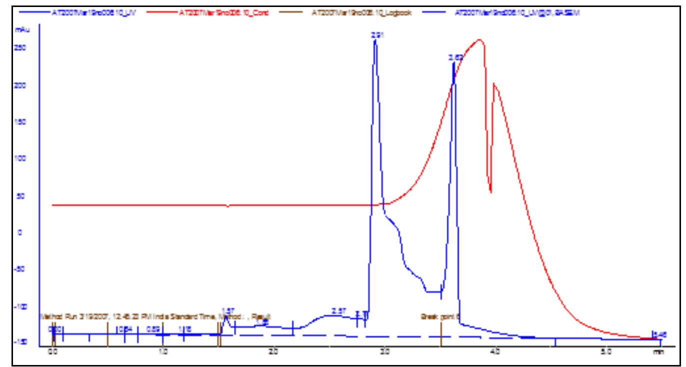

Fig 2: HPLC Chromatogram of Protein Purification of *A. splendens*:

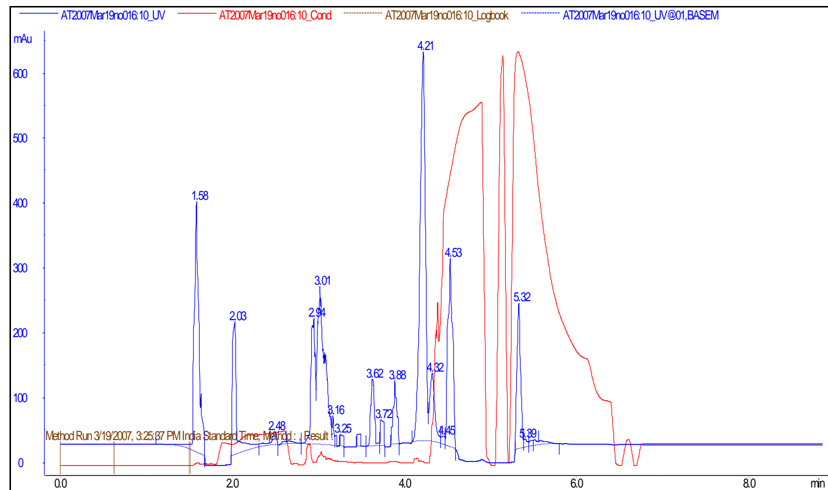

Fig 3: HPLC Chromatogram of Protein Profile of *S. chirayita*:

Table 1: Crude Protein Content of Commercial fish feed, fishery wastes and the Phyto Extracts

S.No	Source	Crude protein content (µg/g)
1	Jet-X fish food	49.6
2	*L. aspera*	97.0
3	*A. splendens*	46.5
4	*S. chirayita*	84.5
5	Fishery wastes	69.4

3.2. Growth study of fish

Increase in the weight and health of fishes were observed in the tanks T2, T3, T4 and T5 compared to commercial feed. Weight of *Carrasius auratus* in various treatments were predicted in the Table-2.Hernandez *et al*., 2007 used soybean meal in the diet of sharpsnout seabream and observed the positive results.

Table 2: Weight of *Carrasius auratus* in various treatments

S. No	Fish Tanks fed with Treatments	Weight of the fish (g)			
		I week	II week	III week	IV week
T1	Control -Commercial fish feed	9.2	9.8	10	10.5
T2	1:1:1 Leucas : Achyranthus : Swertia-(LAS) + 50% fishery wastes	9.5	10.1	10.8	11.9
T3	0.5:1:1 (LAS) + 50% fishery wastes	9.3	9.6	10	11.8
T4	1:0.5:1 (LAS)) + 50% fishery wastes	9.2	9.8	10.1	11.7
T5	1:1:0.5 (LAS)) + 50% fishery wastes	9.2	9.6	10.7	11.8

4. Conclusion

The earlier research studies are well documented with various protein sources and its impact on the fish growth and health. The experiments of Cho 1985; Tacon *et al.,* 1994 ; Bhosale *et al.,* 2010, on the careful formulation and processing fish diets revealed the important role of fish feed in the growth of the aquaculture industry. Hence from the present study it is concluded that the high protein content of *Leucas aspera*, *Achyranthes splendens,* and *Swertia chirayita* and fishery wastes could be used as high protein alternative sources of fish feed. Medicated fish feed curtails the high cost of fish diet and can be used as potential high quality alternative source in the aquaculture industry.

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